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13. ABSTRACT *(Maximum 200 words)*

Polymorphic epithelial mucin (PEM), the product of the MUC1 gene, is expressed in an aberrant form by human breast cancer cells. It is a known, potential target for cytotoxic T lymphocytes of patients with the disease. Cloning of the MUC1 gene and the development of transgenic mice that form human PEM, as well as the genetic modification of mouse breast cancer cells to express human PEM (E3 cells) provides an important model system for the investigation of various means of inducing immunity to PEM. The system mimics as closely as possible the induction of immunity to PEM in breast cancer patients. In the past year, we found that E3 cells modified for the secretion of IL-12 were poorly tumorigenic in MUC1 transgenic mice, and that immunization with X-irradiated IL-12-secreting E3 cells resulted in immunity to breast cancer. We also found that immunization with E3 modified to express B7.1, a co stimulatory molecule required for T cell activation, resulted in prolongation of survival of MUC1 transgenic mice with breast cancer. Autoimmunity failed to develop in the transgenic mice immunized with the MUC-1 positive cells modified to express B7.1. We expanded our study to evaluate the immunotherapeutic benefit of a DNA-based breast cancer vaccine, and are developing transgenic mice that express both alleles of the MUC1 gene.

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PI - Signature 9/25/98 Date

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Progress Report

submitted by Edward P. Cohen, MD and
Joyce Taylor-Papadimitriou, Ph.D.

INTRODUCTION

This investigation was prompted by the finding that polymorphic epithelial mucin (PEM), the product of the MUC1 gene, was expressed in an aberrant form by human breast cancer cells. PEM is a heavily glycosylated macromolecule present on the surface membranes of both normal and malignant cells. The glycosylation pattern of PEM expressed by malignant cells could be distinguished from the glycosylation pattern of PEM expressed by non malignant cells of the same individual. As a consequence, novel T cell epitopes were exposed that were weakly immunogenic as indicated by the finding that the altered PEM was a target for cytotoxic T lymphocytes (CTLs) of the host's immune system. PEM expressed by breast cancer cells has been identified as a weakly immunogenic, tumor associated antigen (TAA). The challenge is to identify an immunotherapeutic approach that can result in the induction of immunity to PEM and rejection of breast cancer cells by breast cancer patients.

One of us (JTP) developed a model system in which human breast cancer associated PEM was expressed in a tissue specific manner by transgenic mice. Like normal, and tumor-bearing patients, the mice were naturally tolerant to human PEM. Analogous to other cellular constituents, the molecule was viewed as "self" by the animal's immune system.

As a model system to investigate therapeutic approaches to induce immunity to PEM in the MUC1 transgenic mice, the MUC1 gene was introduced into 410.4 cells, a breast cancer cell line. (The development of a MUC1 transgenic mouse and a breast cancer cell line modified to express human PEM provide an opportunity to investigate various means of inducing immunity to a known and well characterized

macromolecule expressed by human breast cancer cells. This model system of breast cancer in mice mimics as closely as possible the equivalent disease in patients.) We proposed to test methods found previously to augment the immunogenic properties of TAAs expressed by mouse cancer cells to induce an immune response to human breast cancer-associated mucin in MUC1 transgenic mice naturally tolerant to mucin. The successful application of these techniques could form the basis of an analogous means of inducing immunity to breast cancer-associated mucin in breast cancer patients. The long term objective of our study is the development of a vaccine that can be used in the treatment of breast cancer patients.

At the time of submission of our original proposal, we hypothesized that presentation to the immune system of human breast cancer associated mucin in a microenvironment of immune augmenting cytokines would stimulate an immune response to the breast cancer cells. We also hypothesized that immunization with breast cancer cells further modified to express B7.1, a co stimulatory molecule required for T cell activation, would result in cellular immunity toward the breast cancer cells.

Overview of the Progress Report

The investigation is being carried out in an animal model of breast cancer. The model mimics as closely as possible breast cancer in patients. Transgenic Sac II mice (H-2^k) that express human PEM in a tissue specific manner are used in the studies. As stated, the mice are immunologically tolerant of the macromolecule. E3 cells are a derivative of 410.4 mouse breast carcinoma cells (BALB/c origin, H-2^d) that were genetically modified to express human PEM. The studies are carried out in H-$2^{k/d}$ F1 mice.

The objectives of the first year of the project were accomplished (please see previously submitted progress report). In the second year of the project, Professor Taylor-Papadimitriou and I proposed the following:

1. To compare the survival of MUC1 transgenic mice with breast cancer treated by immunization with cytokine-secreting E3 cells, and to characterize the anti tumor response, and

2. To determine the contribution of B7.1 to the immunogenic properties of mouse breast cancer cells in MUC1 transgenic mice that express human breast cancer associated PEM.

Significant progress toward these and related objectives has been achieved. Data presented in two full manuscripts, one now accepted for publication and "in press," and a second recently submitted for publication are summarized below and indicate the promise of this form of therapy. Our work has been expanded to include a DNA-based vaccine that was used successfully for the induction of immunity to breast neoplasms that occur spontaneously in inbred mice. A third manuscript based on this novel approach has been submitted for publication. The Statement of Work has been revised to reflect these expanded studies.

BODY

Background

The MUC1 gene codes for polymorphic epithelial cell mucin (PEM) which is expressed at the apical surfaces of most glandular epithelial cells. Expression of the gene is dysregulated in breast cancer cells. PEM is dramatically up-regulated and overexpressed in breast (and ovarian) carcinoma cells. The glycosylation pattern is altered, resulting in exposure of novel, potentially immunogenic T cell epitopes.

The extracellular domain of PEM expressed by non malignant cells consists of tandem repeats of twenty amino acids with multiple O-glycans covalently bonded to the amino acid core. The number of repeats in the molecule is polymorphic and is

inherited co dominantly. In breast cancer cells, the composition of the carbohydrate side chains is altered. Cryptic peptides that are ordinarily hidden are expressed by the neoplastic cells. As a consequence, the aberrantly expressed breast cancer-associated mucin can become immunogenic. The altered molecule can become the target of attack mediated by cytotoxic T lymphocytes. The induction of immunity to PEM could have important implications in the treatment of breast cancer patients.

Increasing the immunogenic properties of aberrantly glycosylated mucin expressed by breast cancer cells.

Our objective is use our unique mouse model to investigate methods that will increase the immunogenic properties human breast cancer-associated mucin. The long-term objective is the development of a vaccine that can be used in the treatment of breast cancer patients. Our initial studies were carried out in syngeneic mice injected with a mouse breast cancer cell line (410.4). 410.4 cells are a mouse mammary carcinoma cell line that originated in a BALB/c mouse (H-2^d). The immunogenic properties of 410.4 cells modified to secrete various immune augmenting cytokines were compared. Genes specifying IL-2, interferon-gamma, IL-4 or IL-12 were transfected into 410.4 cells. We then compared the immune responses to 410.4 cells in syngeneic BALB/c mice immunized with the cytokine-secreting cells.

Progress report.

1. Immunity to 410.4 breast carcinoma cells in BALB/c mice immunized with 410.4 cells modified to secrete immune augmenting cytokines.

410.4 cells, an adenocarcinoma breast cancer cell line, is highly tumorigenic in BALB/c mice. One hundred percent of the mice injected into the fat pad of the breast with 410.4 cells formed progressively growing neoplasms that led eventually to the animals' death. Highlights of the effect of IL-12 secretion by the breast cancer cells

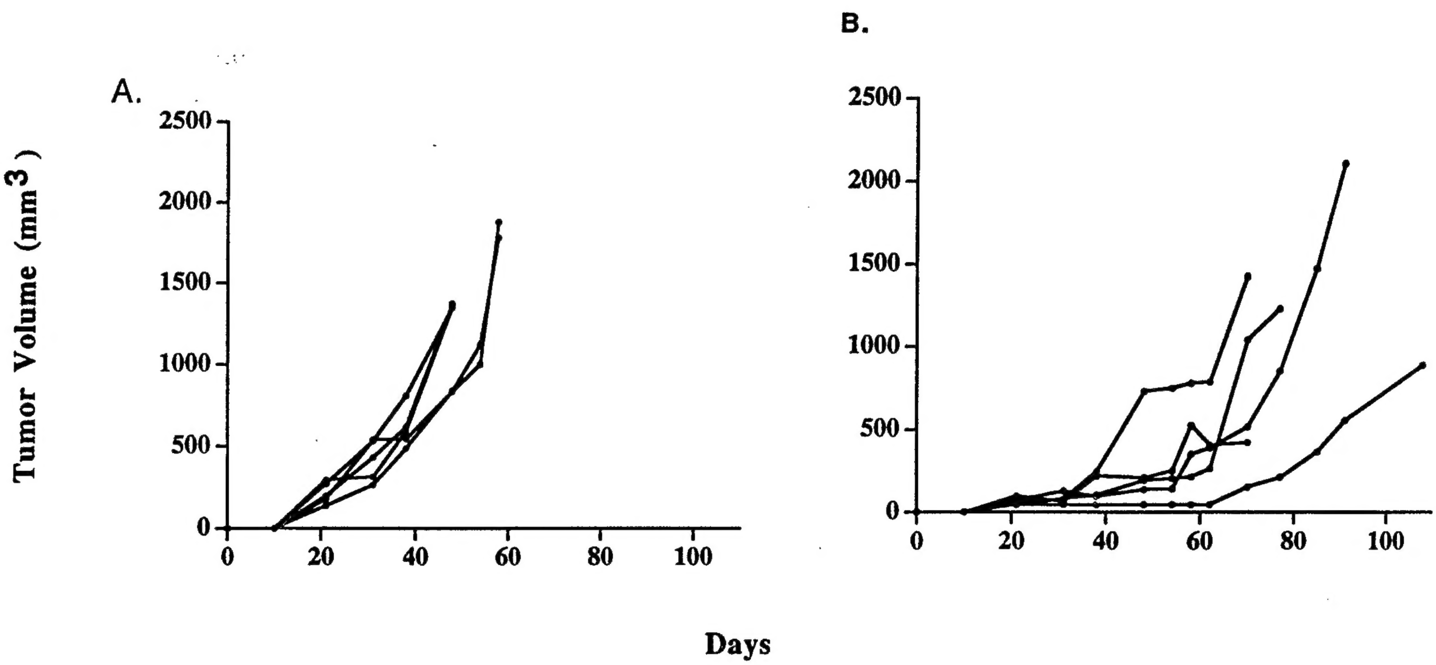
on the cells' immunogenic properties, and of the other cytokines tested, are described, below. Our manuscript describing this work was accepted for publication by the Journal of Immunotherapy and is now "in press." It presents full details of the methods and the results. A copy is included in the Appendix. An overview is as follows:

a. IL-12 formation by 410.4 cells transduced with the retroviral vector, TFG-mIL-12.

TFG-mIL-12, a replication-defective retrovirus, was used to modify 410.4 breast adenocarcinoma cells to form IL-12 (410.4-IL-12). The vector encoded the p40 and p35 heterodimer subunits of IL-12, along with a gene conferring resistance to G418. G418 is an analog of the aminoglycoside antibiotic, neomycin. 410.4 cells from in vitro culture were transduced with the recombinant retrovirus. Afterward, the cells were selected in growth medium containing sufficient quantities (400 ug/ml) of G418 to kill one hundred percent of non transduced cells. The G418-resistant colonies of cells were pooled and maintained as a cell line. An ELISA with specificity for the p70 heterodimer of IL-12 was used to measure IL-12 formation by the transduced cells. The results indicated that 1 X 10^6 G418-resistant cells formed 500 pg IL-12/10^6 cells/48 hrs. Under similar conditions, the culture supernatants of non transduced 410.4 cells, or of 410.4 cells transduced with pZipNeoSV-X, failed to contain detectable quantities of IL-12. Every third passage, the cells were placed in medium containing 400 ug G418. Under these conditions, equivalent quantities of IL-12 were detected when the cells were reanalyzed after three months of continuous culture. An analogous approach was used to modify 410.4 cells to secrete IL-2, IL-4 or interferon-gamma, except that a replication-defective retro virus (pZiPNeoSV(X)) specifying the relevant cytokine gene was substituted for TFG-mIL-12. Like cells modified to secrete IL-12, 410.4 cells transduced with pZiPNeoSV-IL-2, pZiPNeoSV-IL-4 or pZiPNeoSV-interferon-gamma formed the cytokine for more than three months of continuous culture.

To determine the effect of IL-12-secretion by the breast cancer cells on the cells' tumorigenic properties, naive BALB/c mice were injected into the fat pad of the breast with 410.4-IL-12 cells, or, for comparison, with an equivalent number of unmodified 410.4 cells. As indicated (Figure 1), progressively growing tumors formed in both instances. The time to first appearance of tumor, however, was significantly ($P < .01$) delayed in the group of mice injected with 410.4-IL-12 cells. The delayed appearance of the tumor was reflected in the prolonged survival of BALB/c mice injected with 410.4-IL-12 cells. Mice injected with 410.4-IL-12 cells survived significantly ($P < .001$) longer than mice injected with an equivalent number of non IL-12-secreting 410.4 cells (92 ± 14 days and 56 ± 7 days respectively). Eventually, mice in both groups died from progressive tumor growth.

Figure 1.



Legend: Tumor growth in BALB/c mice inejcted with 410.4-IL-12 cells.

Naive mice were injected into the fat pad of the breast with 1 X 10^6 410.4-IL-12 cells or wiith an equivalent number of unmodified 410.4 cells. Each line represents tumor growth in an individual mouse. The endpoint of the line represents death of the mouse. A = injected with unmodified cells B= injected with 410.4-IL-12 cells

One possible explanation for the delayed appearance of tumors in BALB/c mice injected with 410.4-IL-12 cells is that expression of neomycin phosphotransferase, a xenoantigen, contributed to the cells' immunogenic properties. This point was investigated by comparing the survival of BALB/c mice injected with 410.4 cells transduced with pZipNeoSV(X), a retro viral vector that specified neomycin phosphotransferase, but lacked a cytokine gene, with the survival of mice injected with an equivalent number of 410.4 cells modified to secrete IL-12. In the experiment, naive mice were injected into the fat pad of the breast with 1 X 10^6 410.4-IL-12 cells, or with neomycin-resistant 410.4 cells transduced with pZipNeoSV(X). As an additional control, other naive mice were injected with the same number of non transduced 410.4 cells. The results indicated that the median survival of mice injected with cells transduced with pZipNeoSV(X), 65 ± 15 days, was not significantly different than the survival of mice injected with unmodified 410.4 cells alone (60 ± 11) days ($p = .1$). Survival of mice in both groups was significantly ($P < .001$) less than that of mice injected with an equivalent number of 410.4 cells modified for IL-12-secretion (94 ± 19) days.

Conceivably, differences in rate of proliferation of 410.4, 410.4 cells transduced with pZipNeoSV(X) and 410.4-IL-12 cells accounted for the delayed first appearance of tumor, and prolonged survival of mice injected with 410.4-IL-12 cells. This point was investigated by measuring the generation times of the various cell types. The results indicated that the doubling time of the cells, approximately 36 hrs., was essentially the same for each cell type.

<u>Progressively growing neoplasms formed in BALB/c nude mice injected with 410.4-IL-12 cells.</u>

As indicated, BALB/c mice injected with 410.4 cells modified to secrete IL-12 survived significantly longer than BALB/c mice injected with an equivalent number of unmodified 410.4 cells, although the animals in both groups died from progressive tumor growth. The prolonged survival of mice injected with 410.4-IL-12 cells may have been an indication the cells' immunogenic properties had been partially enhanced by the secretion of IL-12.

To investigate this question, we injected BALB/c nude mice with 410.4-IL-12 cells, or 410.4 cells, and compared the time to first appearance of tumor, and survival of mice in the two groups. Unlike immunocompetent BALB/c mice, the time to first appearance of tumor, and survival of BALB/c nude mice injected with 410.4-IL-12 cells, or 410.4 cells, were not significantly different. The median survival time, 46 ± 4 days for mice injected with 410.4-IL-12 cells was approximately the same as the median survival time of mice injected with unmodified 410.4 cells, (43 ± 9 days; P = .32). The results were consistent with an enhancement of the immunogenic properties of 410.4 cells that had been modified to secrete IL-12.

<u>Progressively growing neoplasms formed in immunocompetent BALB/c mice injected with a mixture of 410.4-IL-12 cells and 410.4 breast carcinoma cells.</u>

The prolonged period of survival of BALB/c mice injected with 410.4 cells modified to secrete IL-12 suggested that IL-12 had enhanced the immunogenic properties of the breast cancer cells. This point was investigated by comparing time to first appearance of tumor, and survival, of BALB/c mice injected into the fat pad of the breast with a mixture of 410.4 cells and 410.4-IL-12 cells with the time to first appearance of tumor, and survival, of BALB/c mice injected with an equivalent number of 410.4 cells alone. The results indicated that the time to first appearance of tumor in mice injected with the mixture of 410.4-IL-12 cells and 410.4 cells was not significantly different than the time to first appearance of tumor in mice injected

with 410.4 cells alone. At the same time, the median survival of mice injected with the mixture of 410.4-IL-12 cells and 410.4 cells was not significantly different than the median survival of mice injected with 410.4 cells alone (MST = 42 ± 13 days and 60 ± 5 days respectively, p = .06).

Thus, if modification of 410.4 cells to secrete IL-12 stimulated an immune response toward 410.4 cells, the response was insufficient to inhibit growth of the breast cancer cells.

One possible explanation for the failure of 410.4-IL-12 cells to control tumor growth is that 410.4 cells secrete immunosuppressive factors that inhibited cell mediated immune responses. This point was investigated by adding culture supernatants from 410.4 cells to a mixed cell culture consisting of spleen cells from naive BALB/c mice (H-2^d) and allogeneic LM fibroblasts (H-2^k). The allogeneic fibroblasts were mitomycin-C-treated (50 ug/ml; 30 min., 37°) before they were added to the spleen cell-suspension from BALB/c mice. Spleen cell proliferation was determined by the addition of ^{3}H-thymidine to the mixed cell cultures for the last 18 hrs. of the incubation, followed by measurements of the incorporation of ^{3}H-thymidine by the proliferating cells. The results indicated that the addition of culture supernatants of 410.4 cells reduced the proliferative response by approximately five fold (2142 ± 71 cpm in the presence of supernatant vs. 10664 ± 313 in the absence of supernatant). The data were consistent with the secretion of undefined immunosuppressive factors by the breast cancer cells.

<u>Partial immunity toward 410.4 breast cancer cells was generated in BALB/c mice injected with X-ray-inactivated 410.4-IL-12 cells.</u>

The delayed appearance of tumor and prolonged survival of BALB/c mice injected with 410.4-IL-12 cells suggested that the weakly immunogenic properties of 410.4 cells had been enhanced by IL-12-secretion, but that the enhanced immunogenic properties were insufficient to control tumor growth. Inactivation of the cells by X-

irradiation might preserve the cells' immunogenic properties as it prevented the cells from dividing. This point was investigated by first immunizing naive BALB/c mice with X-irradiated 410.4-IL-12 cells and then challenging the mice with an injection of 410.4 cells. In the experiment, the mice were injected s.c. three times with 1 X 10^6 X-irradiated (5000 rads from a ^{60}Co source) 410.4-IL-12 cells at monthly intervals. Thirty days after the last injection, the mice were injected into the fat pad of the breast with 1 X 10^6 410.4 cells. As controls, naive BALB/c mice were injected according to the same schedule with equivalent numbers of X-irradiated 410.4 cells, or with X-irradiated, non IL-12-secreting 410.4 cells transduced with the vector, pZipNeoSV(X). The results indicated that one hundred percent of the mice injected with the irradiated 410.4-IL-12 cells, followed by the challenging injection of 410.4 cells, survived indefinitely, more than 90 days. The mice appeared to have rejected the breast cancer cells. Under similar conditions, none of the mice injected with X-irradiated 410.4 cells, or with 410.4-SV-X cells, followed by the challenging injection of 410.4 cells, developed immunity to the tumor. They died from progressive tumor-growth.

Immunizations with X-irradiated 410.4-IL-12 cells induced long-term immunity to 410.4 cells in BALB/c mice. Mice treated by immunization with X-irradiated 410.4-IL-12 cells that survived the first injection of 410.4 cells were resistant when rechallenged by a second injection of 410.4 cells. They survived significantly ($P < .001$) longer than naive mice injected with 410.4 cells alone.

These data are presented in a more complete form in the manuscript from our lab by Victoria Carr-Brendel, Dubravka Markovic, Michael Smith, Joyce Taylor-Papadimitriou and Edward P. Cohen entitled, **Immunity to Breast Cancer in Mice Immunized with X-Irradiated Breast Cancer Cells Modified to Secrete IL-12** which has been accepted for publication by the *Journal of Immunotherapy* and is now "in press." A copy of the manuscript is included in the Appendix. The results indicate the immunotherapeutic properties of X-irradiated breast cancer cells modified to secrete IL-12.

2. Immunity to Murine Breast Cancer Cells Modified to Express MUC1, a Human Breast Cancer Antigen.

The cloning and sequencing of MUC1, a gene specifying polymorphic epithelial mucin (PEM), by Dr. Taylor-Papadimitriou and her colleagues, enables studies in a murine model of the immunogenic properties of a human breast cancer antigen.

E3 cells are a derivative of 410.4 cells. The cells were modified by transfection with MUC1 to express PEM, a human breast cancer associated mucin. Whether or not the human mucin was antigenic in BALB/c mice had not been determined. To investigate this question, tumor growth was measured in naive BALB/c mice injected into the fat pad of the breast with E3 cells, or, for comparison, with an equivalent number of unmodified 410.4 cells. The results indicated that tumor growth occurred in both instances. It led to the animals' death. However, the first appearance of tumor in mice injected with E3 cells was significantly delayed, relative to that of mice injected with 410.4 cells. It suggested that human MUC1 was insufficiently immunogenic in mice to induce protective immunity toward E3 cells.

Figure 1. Tumor growth in BALB/c mice injected into the breast with 410.4 or E3 cells.

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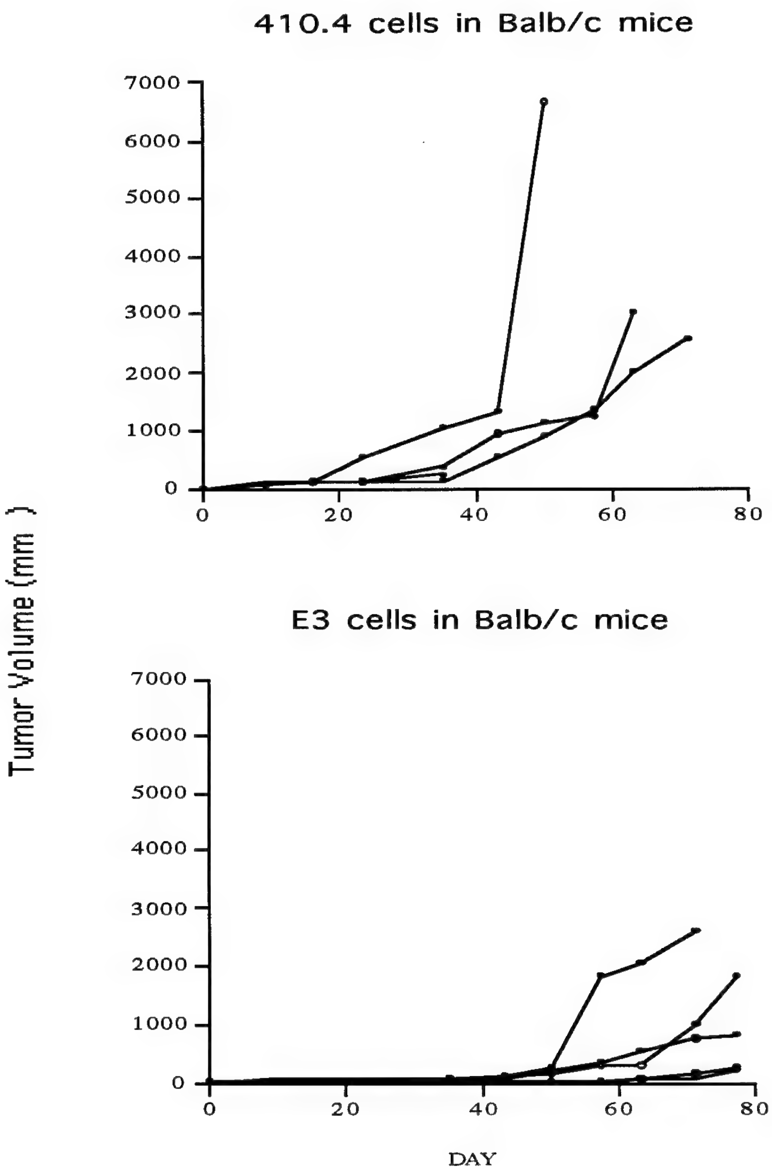

Legend: Tumor growth was measured in naive BALB/c mice injected into the fat pad of the breast with 1 x 10^6 E3 cells (B) in a total volume of 200 ul growth medium. For comparison, naive mice were injected with 1 x 10^6 410.4 cells (A) in 200 ul growth medium. Two dimensional tumor measurements were performed and the volume of the tumor was calculated by standard methods. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

An analogous study was carried out to measure tumor growth in transgenic mice modified to express MUC1. The results (Figure 2) indicated that like to delayed appearance of tumor in BALB/c mice injected with E3 cells, the first appearance of tumor was delayed in MUC1 transgenic mice injected with the cells that were modified to express human PEM. The growth of E3 cells in the transgenic mice led, eventually, to the animals' death.

Figure 2. Tumor growth in MUC1Tg X BALB/c mice injected into the breast with 410.4 cells or E3 cells.

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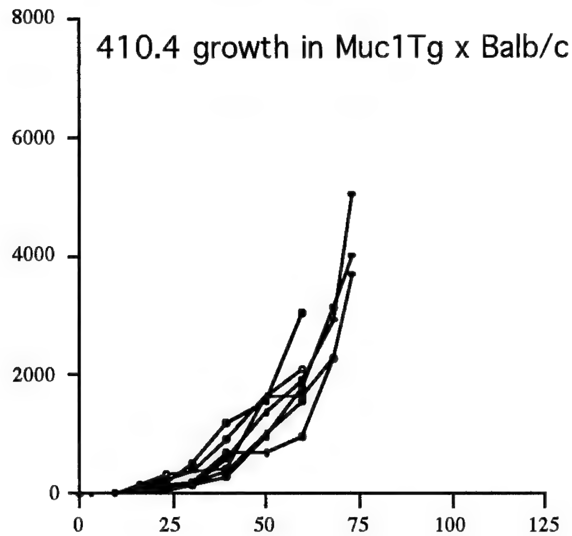

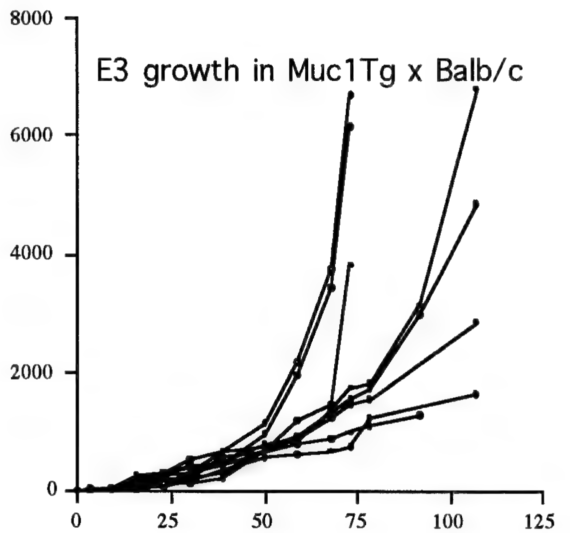

Legend: Tumor growth was measured in naive BALB/c x MUC-1 Tg F1 mice injected into the fat pad of the breast with 1 x 10^6 E3 cells (B) in a total volume of 200 ul growth medium. For comparison, naive mice were injected with 1 x 10^6 410.4 cells (A) in 200 ul growth medium. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

With this background information, we next investigated the effect of modifying E3 cells to secrete various immune augmenting cytokines on the cells' immunogenic properties.

Modification of E3 cells to secrete IL-12.

As described above, we used retroviral transduction to modify E3 breast carcinoma cells to form IL-2, IL-4, IL-12 or interferon gamma. After confirmation of cytokine-secretion by the genetically modified cells, we followed a similar approach to determine the cells' tumorigenic properties in transgenic mice that expressed human PEM as self.

The results were as follows:

Figure 3. Tumor growth in BALB/c F1 x MUC-1 Tg mice injected into fat pad of the breast with E3-IL-12, E3-SV-X, E3-IFN-g, E3-IL-2, E3-IL-4, or unmodified E3 cells.

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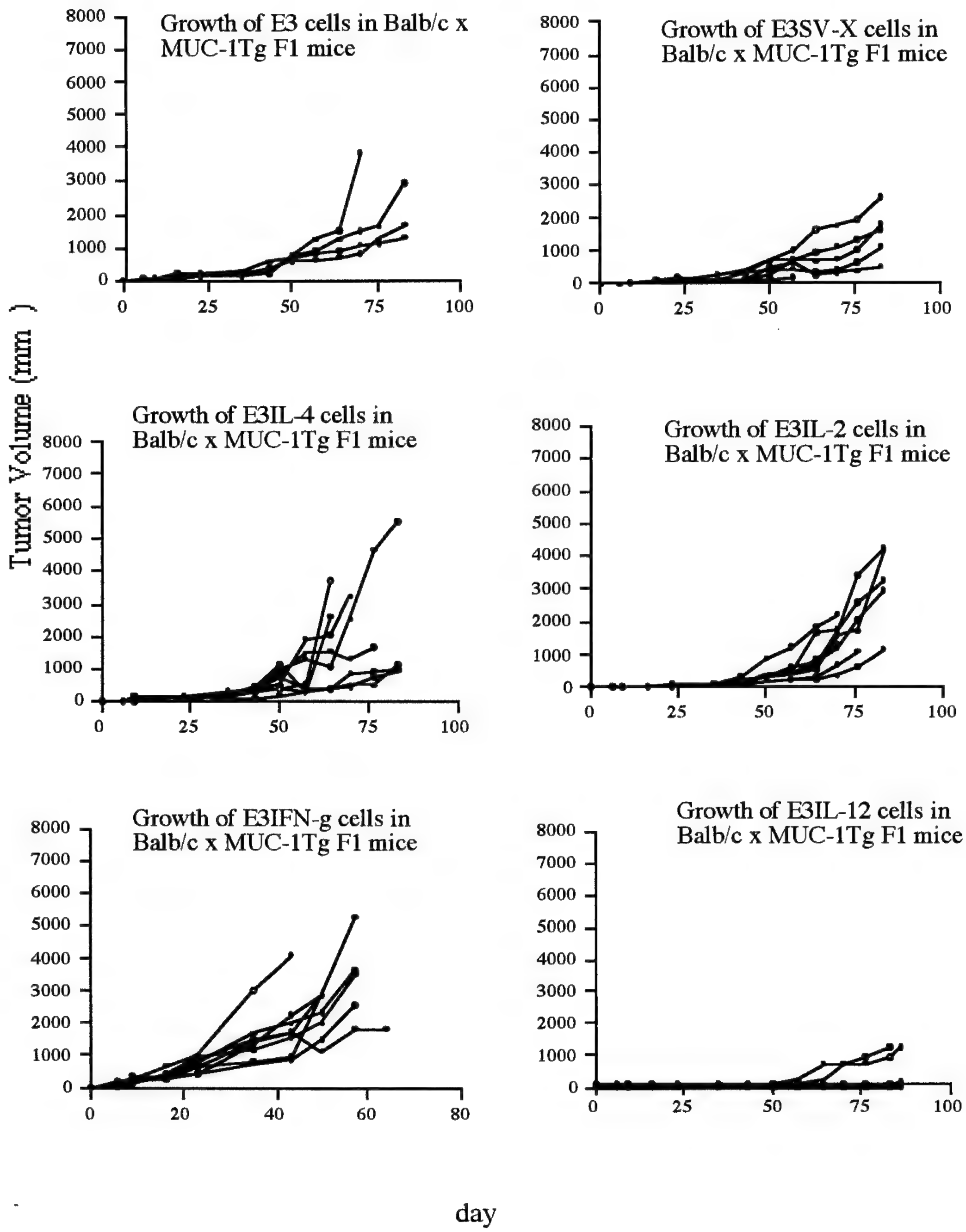

Legend: Naive BALB/c x MUC-1 Tg F1 mice were injected into the fat pad of the breast with 1 x 10^6 E3 cells Thai had been modified for cytokine secretion. For comparison, other naive mice were injected with an equivalent number of non cytokine-secreting E3 cells. Each cell-type was suspended in 200 ul of growth medium for the injection. Two dimensional tumor measurements were performed and the volume of the tumor was calculated according to standard methods. Each

line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

The results indicate that like 410.4 cells modified to secrete IL-12, the cytokine that had the most profound effect on the immunogenic properties of E3 cells was IL-12-secretion. Some of the transgenic mice injected with E3-IL-12 cells appeared to have rejected the breast cancer cells and survived indefinitely.

In the forthcoming year, we plan to investigate the immunotherapeutic properties of E3-IL-12 cells against the growth of E3 cells in the MUC1 transgenic mice. The success of these studies could lead to an analogous form of therapy of breast cancer patients.

Expression of B7.1 by 410.4 cells modified to express human mucin (E3 cells) augments the cells' immunogenic properties and fails to induce autoimmunity in MUC1 transgenic mice.

As noted previously, PEM, the product of the MUC1 gene is expressed on the apical surfaces of most normal glandular epithelial cells. Its lubricant properties perform important physiological functions. Mucin is over expressed and aberrantly glycosylated by breast cancer cells. It is a potential target of immune-mediated attack.

The development of a human MUC1 transgenic mouse that expresses PEM in a tissue specific manner, along with the genetic modification of 410.4 mouse mammary carcinoma cells to express human PEM (E3 cells) provides a unique opportunity to investigate the effects of B7.1 on the immunogenic properties of E3 cells. B7.1 is a co stimulatory molecule required for T cell activation. It is constitutively expressed by dendritic cells and other types of professional antigen presenting cells. Conceivably, expression of B7.1 by E3 cells could augment the cells' immunogenic properties in MUC1 transgenic mice.

To investigate this question, E3 cells were modified to express B7.1. After confirmation of expression of B7.1, the modified cells were tested for their tumorigenic properties in MUC1 transgenic mice.

The results indicated the following:

1. Expression of B7.1 by E3 mouse mammary carcinoma cells resulted in a dramatic inhibition of tumor growth in MUC1 transgenic mice. The first appearance of tumor was delayed and the mice survived significantly longer than mice in various control groups.

2. The inhibition of tumor growth was directly related to the level of expression of B7.1 by the breast cancer cells. The tumorigenic properties of E3 cells that expressed high levels of B7.1 were less than that of cells that expressed low levels of B7.1.

3. B7.1 expression by E3 cells had no effect on tumor growth in immune deficient nu/nu (nude) mice, suggesting that cytotoxic T lymphocytes were involved in mediating resistance to tumor growth.

4. The activity of both $CD4^+$ and $CD8^+$ cells was required for resistance to growth of the breast cancer cells in the transgenic mice.

5. Transgenic mice injected with E3 cells modified to express B7.1 failed to exhibit evidence of autoimmune disease. There was no evidence of lymphocytic infiltration or organ degeneration in transgenic mice that rejected the modified cells. The survival of the mice was indistinguishable from that of non injected transgenic mice.

These data are presented in a more complete form in the enclosed manuscript by M Smith, JM Burchell, R Graham, EP Cohen and J Taylor-Papadimitriou entitled,

"Expression of B7.1 in a MUC1 expressing mouse mammary epithelial tumour cell line overcomes tolerance but does not induce autoimmunity in MUC1 transgenic mice." The manuscript was recently submitted for publication.

Taken together, these findings indicate the promise of a MUC1-based tumor vaccine in the treatment of breast cancer patients.

Additional studies

1. Treatment of Breast Cancer with a DNA-based Breast Cancer Vaccine

During the course of these investigations, we carried out additional, complementary studies designed to investigate the immunotherapeutic properties of a DNA-based breast cancer vaccine. The vaccine was prepared by transfection of a mouse fibroblast cell line with DNA from an adenocarcinoma of the breast that formed spontaneously in a C3H/He mouse. The results are summarized in the enclosed manuscript entitled, "Treatment of Breast Cancer with Fibroblasts Transfected with DNA from Breast Cancer Cells," which has been submitted for publication (a copy of the manuscript is included in the Appendix).. They indicate the promise of this novel form of breast cancer therapy.

The underlying rationale of the DNA-based vaccine is that the immunogenic properties of breast cancer-associated antigens (TAAs), the products of mutant or dysregulated genes in the malignant cells, can be enhanced if the genes are expressed by highly immunogenic cells. To investigate this question, we first modified LM mouse fibroblasts (H-2^k) to secrete interleukin-2 (IL-2). (LM mouse fibroblasts form B7.1 constitutively. The cells act as efficient, professional antigen presenting cells.) Afterward, the modified fibroblasts were co transfected with DNA from the spontaneous breast neoplasm, along with a plasmid (pHyg) that conferred resistance to hygromycin, used for selection. Pooled colonies of hygromycin-resistant, transfected cells were then tested in C3H/He mice for their

immunotherapeutic properties against the growth of the breast cancer cells. The results indicated that tumor-bearing mice treated by immunization with the transfected cells survived significantly ($p < .01$) longer than mice in various control groups including mice injected with breast cancer cells and non DNA transfected fibroblasts. Similar beneficial effects were seen in C57BL/6J mice injected with a syngeneic breast carcinoma cell line (E0771) and modified fibroblasts transfected with DNA from E0771 cells. The immunity was mediated by $CD8^+$ T cells since immunized mice depleted of $CD8^+$ cells failed to resist tumor growth. The data indicate the promise of a vaccine effective in the treatment of breast cancer prepared by transfection of breast cancer DNA into a highly immunogenic, fibroblast cell line.

The use of a DNA-based tumor vaccine has several important advantages: Since the transfected DNA is integrated by the fibroblasts, and is replicated as the cells divide, only a small quantity of DNA is required to prepare the vaccine. Sufficient DNA could be obtained from small surgical specimens (a needle biopsy would provide enough DNA for this purpose) or from microscopic amounts of tumor. The transfected cells proliferate in vitro under standard cell culture conditions. Thus, the number of transfected cells can be expanded as might be required for multiple immunizations of the breast cancer patient.

In other instances, defined tumor antigens or unfractionated tumor peptides have been used for tumor immunotherapy. However, few defined tumor antigens have been identified and cloned, and immunization with unfractionated tumor peptides requires large amounts of tumor if multiple immunizations are to be carried-out. Sufficient quantities of tumor tissue may not be available if patients are in clinical remission.

Another approach is to "feed" cloned tumor-antigens or protein-extracts of cancer cells to antigen-presenting cells. This approach has certain advantages. Defined tumor antigens can substitute for tumor tissue, enabling patients with low tumor

burdens to receive therapy. Since the cloned antigens used for immunotherapy are defined, irrelevant antigens can be avoided and the likelihood of toxic responses in the immunized patient may be reduced. However, there are concerns. Immune therapy of cancer patients with cloned tumor antigens may be limited to a small number of tumors for which defined antigens have been identified. In addition, some tumor cells within the malignant cell-population may express tumor antigens other than used in the immunization protocol. Immunization with defined antigens increases the chance that antigen-loss escape mutants will survive and continue to proliferate in the patient. Other experimental approaches such as "feeding" dendritic cells with protein extracts of cancer cells require large amounts of tumor, and the isolation of autologous dendritic cells . Patients in clinical remission may not have sufficient amounts of tumor to prepare the vaccine. The isolation of autologous dendritic cells requires leukophoresis and repeated rounds of therapy may not always be possible.

2. Development of a transgenic mouse that expresses both alleles of human MUC1.

SacII homozygote MUC1 transgenic mice are H-2^k. SacII x BALB/c F1 mice were used in the experiments involving E3 cells (of BALB/c mouse origin.) MUC1 in humans is the product of two alleles. Thus, a transgenic mouse that expresses both alleles of human MUC1 would represent a model system of breast cancer therapy that more closely represents the disease in patients.

To investigate immune responses to PEM when both MUC1 alleles are expressed, we have backcrossed SACII mice onto BALB/c and C57BL stains of mice. Although the offspring of the first backcross are transgenic, subsequent backcrossings were carried out followed by analysis of DNA from tail snips to detect mice that expressed human MUC1. Eleven backcrosses were performed, and the heterozygotes within the latest offsprings are now being cross to identity homozygotes in the two strains.

3. Development of mice expressing MUC1 and a human class I allele.

MUC1 transgenic mice allow experiments that will allow means of overcoming tolerance to a self tumor associated antigen (PEM) without the induction of autoimmunity. However, the immune response in the mice will depend on the immune repertoire. We have transgenic mice which express the human HLA A2001 allele as a fusion protein with the mouse a3 domain, to facilitate interaction with mousse T cells. We now plan to cross these mice with MUC1 transgenic mice on a C57BL/6 background. RMA cells modified for cytokine secretion that are then transfected with the gene for MUC1 will be used to investigate induction of an immune response to PEM.

CONCLUSIONS

We conclude the following:

1. Immunization with X-irradiated 410.4 cells modified to secrete IL-12 prolonged the survival of mice with breast cancer.

2. E3 cells modified for the secretion of IL-12 were poorly tumorigenic in MUC1 transgenic mice. Like 410.4 cells modified to secrete IL-12 cells, immunization with E3-IL-12 cells may induce immunity to E3 cells in MUC1 transgenic mice.

3. Immunization with breast cancer cells modified to express B7.1, a co stimulatory molecule, resulted in immunity to breast cancer in MUC1 transgenic mice, and prolonged survival of mice with breast cancer.

4. Autoimmunity failed to develop in MUC1 transgenic mice treated by immunization with MUC-1 expressing breast cancer cells modified to express B7.1, pointing toward the safety of the genetically modified vaccine.

5. Treatment of mice with breast cancer with a DNA-based breast cancer vaccine led to breast cancer immunity and prolonged survival. A vaccine prepared by transfer of breast cancer DNA into a highly immunogenic cell line has important advantages. It can be a practical, additional means of vaccine preparation.

Appendix 7

STATEMENT OF WORK (REVISED)

1. Introduction of genes for various cytokines into E3 cells. Selection of genetically modified cells. Assays for cytokine-secretion (completed)

1a. Introduction of the genes for B7.1 into E3 cells. Selection of genetically modified cells. Assays for B7.1 expression. (completed)

2. Comparison of the survival of MUC1 transgenic mice immunized with cytokine-secreting E3 cells challenged with viable E3 cells. Characterization of the anti tumor response (in progress)

2a. Determination of the contribution of B7.1 to the immunogenic properties of mouse breast cancer cells modified to express human PEM in MUC1 transgenic mice (completed)

3. Comparison of the survival of MUC1 transgenic mice bearing PEM-positive breast cancers of varying sizes treated with cytokine-secreting E3 cells (12 months)

3a. Development of a construct that specifically restricts expression of B7.1 to breast cancer cells (12 months)

4. Detection of an autoimmune response in transgenic mice immunized with breast cancer cells modified to express human PEM and B7 (completed)

5. Development of a DNA-based vaccine breast cancer vaccine that prolongs survival of mice with small breast neoplasms (completed)

6. Determination of the maximum immunotherapeutic benefit of the DNA-based breast cancer vaccine in mice with breast cancer (18 months)

7. Development of a MUC1 transgenic mouse that expresses both human MUC1 alleles (18 months)

8. Development of transgenic mice that express human MUC1 and a human class I MHC allele. (18 months)

Immunity to Breast Cancer in Mice Immunized with X-Irradiated Breast Cancer Cells Modified to Secrete IL-12.

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ABSTRACT

A mouse mammary adenocarcinoma cell line (410.4) originating in a BALB/c mouse, was transduced with a retroviral vector (TGF-mIL-12-Neo) that encoded murine IL-12. After confirmation of IL-12-secretion, the cells were tested for their tumorigenic properties in BALB/c mice. The results indicated that unlike other tumors modified for cytokine-secretion, modification of 410.4 cells to secrete IL-12 (410.4-IL-12 cells) failed to eliminate the cells' neoplastic growth properties. Progressively growing tumors of 410.4-IL-12 cells invariably formed in syngeneic BALB/c mice that led, eventually, to the animals' death. However, the cells' immunogenic properties were preserved as indicated by the finding that immunizations with 410.4-IL-12 cells, inactivated before injection by X-irradiation, resulted in potent, long-term immunity toward unmodified 410.4 cells, and protected the mice against the malignant proliferation of the breast cancer cells. We conclude that modification of 410.4 cells for IL-12-secretion augmented the response of syngeneic BALB/c mice to weakly immunogenic tumor associated antigens expressed by the cells. The increase in the cells' immunogenic properties, however, was insufficient to prevent tumor growth in the mice. The results point toward the immunotherapeutic potential of X-irradiated tumor cells modified for the secretion of immune augmenting cytokines.

Key Words

Breast Cancer, Immunotherapy, Immunity, IL-12, Mice.

INTRODUCTION

The tumorigenic properties of highly malignant cells can be altered if the cells are genetically modified to secrete immune-augmenting cytokines. In experimental systems, neoplastic cells modified to secrete IL-2 (1-5), IL-4 (6,7), GM-CSF (8,9), or interferon-gamma (10, 11) were rejected, primarily by cellular immune mechanisms. Animals rejecting the cytokine-secreting tumor cells exhibited strong anti tumor immune responses that in some instances resulted in tumor cell rejection. Under analogous circumstances, unmodified tumor cells were insufficiently immunogenic and grew progressively. Immunization with cytokine-secreting tumor cells is under active investigation as a new, and important novel means of tumor therapy (12).

Like other immune augmenting cytokines, modification of tumor cells to secrete IL-12 also affected the cells' tumorigenic properties (13-17). Immunizations of mice with IL-12-secreting sarcoma cells (13), melanoma (14) and other types of tumors (15-17) resulted in rejection of the IL-12-secreting cells and the induction of T cell-mediated anti tumor immune responses that were directed toward the unmodified tumor as well. As an indication of the potential importance of this form of cancer therapy, mice immunized with the IL-12-secreting tumor cells, challenged by injection of viable, unmodified cells, survived significantly longer than naive mice injected with unmodified tumor cells alone.

Here, we report the results of treating mice with a mouse mammary carcinoma cell line (410.4), modified by retroviral transduction to secrete IL-12. 410.4 cells were derived from a spontaneously occurring mammary carcinoma arising in a BALB/c mouse (18). The tumor cells were highly aggressive in syngeneic mice. They metastasized early, and secreted immunosuppressive factors, such as prostaglandin-E2 that inhibited IL-12 secretion (18-21). Unlike other tumors modified for cytokine-secretion, however, we found that modification of 410.4 cells to secrete IL-12 (410.4-IL-12 cells) failed to eliminate the cells' tumorigenic properties. Tumors of 410.4-IL-12 cells invariably formed in syngeneic BALB/c mice. However, immunizations with 410.4-IL-12 cells, inactivated before injection by X-irradiation, generated immunity toward unmodified 410.4 cells. Mice rejecting X-irradiated 410.4-IL-12 cells were protected against the malignant proliferation of the breast cancer cells. Under similar conditions, anti tumor immune responses failed to develop in mice immunized with non IL-12-secreting 410.4 cells subjected to equivalent amounts of X-irradiation.

MATERIALS AND METHODS

Experimental animals and tumor cell lines

Six to eight week old BALB/c mice were obtained from Charles River Laboratory (Wilmington MA, USA). BALB/c nu/nu (nude) mice were from Jackson Laboratories (Bar Harbor, ME, USA). The animals were housed in our animal maintenance facility in accordance with the NIH guide for the Care and Use of Laboratory Animals.

410.4 breast adenocarcinoma cells were obtained from Joyce Taylor-Papadimitriou. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO), 1 mM sodium pyruvate, and 100 units/ml penicillin and 100 ug/ml streptomycin (Gibco/BRL, Grand Island, NY) (growth medium), under standard cell culture conditions (37^oC in a humidified 7% CO_2/air atmosphere). LM cells, a mouse fibroblast cell line of C3H/He mouse origin ($H\text{-}2^k$), were from the American Type Culture Collection (ATCC). They were maintained in growth medium under standard cell culture conditions.

Modification of 410.4 cells for IL-12-secretion.

A retroviral vector (TFG-mIL-12) (22), kindly provided by H. Tahara, (University of Pittsburgh, Pittsburgh, PA) was used to modify 410.4 cells to secrete IL-12. The vector specified the murine heterodimeric subunits (p40 and p35) of IL-12, and a gene conferring neomycin resistance. Both were under control of the TFG vector 5' LTR. 410.4 cells were transduced according to the protocol

described by Tahara et al., (15). In brief, 5 X 10^5 410.4 cells were added to individual wells of a 6 well plate (Falcon). Twenty four hours later, 1 ml of TFG-mIL-12-Neo retroviral supernatant in the presence of polybrene (8 ug/ml) was added to each well. The cells were maintained in growth medium containing 400 ug/ml of the neomycin analog, G418. After a 14 day period of incubation, cells proliferating in the G418-containing medium were pooled and maintained as a cell line. As a control, the same protocol was followed except that a plasmid (pZipNeoSV-X, from M.K.L. Collins, University College, London, England) was substituted for TFG-mIL-12 (410.4-SV-X cells). (pZipNeoSV-X specifies a neomycin resistance gene, but not the gene for IL-12.) Lipofectin™ (Gibco/BRL, Grand Island, New York) was used to facilitate uptake of pZipNeoSV-X by 410.4 cells, according to the manufacturer's instructions.

Detection of IL-12 formation by 410.4 cells transduced with TFG-mIL-12.

An ELISA (Endogen, Woburn, MA) was used to detect the formation of IL-12 by 410.4 cells transduced with TFG-mIL-12. Cell culture supernatants were analyzed for the p70 heterodimer of IL-12. The ELISA was not cross-reactive with the p40 monomer or homodimer. In brief, 1 X 10^6 cells 410.4-IL-12 cells were added to cell culture flasks containing 10 ml of RPMI medium (Gibco) supplemented with 2.0 mM Na pyruvate, 10 percent fetal bovine serum (Sigma), 0.15 M Hepes and antibiotics. After incubation for 48 hrs. under standard cell culture conditions, the culture supernatant was collected and

passaged through a 0.45 u nitrocellulose filter before the assay for IL-12 was performed. The same protocol was used to analyze the culture supernatants of 410.4 cells transduced with pZipNeoSV-X, (410.4-SV-X) and non transduced 410.4 cells.

Growth of 410.4-IL-12 cells in BALB/c or BALB/c nude mice.

Two dimensional measurements were used to measure the growth of tumor in BALB/c or BALB/c nude mice injected with 410.4-IL-12 cells. The mice were injected into the fat pad of the breast with 1 x 10^6 410.4-IL-12, 410.4-SV-X or 410.4 cells suspended in 200 ul of growth medium. Tumor growth was estimated grossly, using an vernier caliper to obtain the two dimensional measurements. The volume of the tumor was calculated by the formula $0.4ab^2$, where a = length and b = width of the tumor (23).

RESULTS

1. IL-12 formation by 410.4 cells transduced with the retroviral vector, TFG-mIL-12.

TFG-mIL-12, a replication-defective retrovirus, was used to modify 410.4 breast adenocarcinoma cells to form IL-12. The vector encoded the p40 and p35 heterodimer subunits of IL-12, along with a gene conferring resistance to the aminoglycoside antibiotic, neomycin-analog, G418. 410.4 cells from in vitro culture were transduced with the virus, and then selected in growth medium containing sufficient quantities (400 ug/ml) of G418 to kill one hundred percent of non transduced cells. The antibiotic-resistant colonies of cells were pooled and maintained as a cell line. An ELISA with specificity for the p70 heterodimer of IL-12 was used to measure IL-12 formation by the transduced cells. The results indicated that 1 X 10^6 G418-resistant cells formed 500 pg IL-12/10^6 cells/48 hrs. Under similar conditions, the culture supernatants of non transduced 410.4 cells, or 410.4 cells transduced with pZipNeoSV-X failed to contain detectable quantities of IL-12. Every third passage, the cells were placed in medium containing 400 ug G418 for the subsequent two passages, after which the cells were placed in growth medium. Under these circumstances, equivalent quantities of IL-12 were detected when the cells were reanalyzed after three months of continuous culture (these data are not presented).

2. Progressively growing neoplasms formed in BALB/c mice injected into the fat pad of the breast with 410.4-IL-12 cells.

410.4 cells, an adenocarcinoma breast cancer cell line, is highly tumorigenic in syngeneic BALB/c mice. One hundred percent of the mice injected into the fat pad of the breast with 410.4 cells formed progressively growing neoplasms that led eventually to the animals' death.

To determine if modification of the breast cancer cells to form IL-12 affected the cells' tumorigenic properties, naive BALB/c mice were injected into the fat pad of the inguinal breast with 410.4-IL-12 cells, or, for comparison, with an equivalent number of unmodified 410.4 cells. As indicated (Figure 1), progressively growing tumors formed in both instances. The time to first appearance of tumor, however, was significantly ($P < .01$) delayed in the group of mice injected with 410.4-IL-12 cells. The delayed appearance of the tumor was reflected in the prolonged survival of mice injected with 410.4-IL-12 cells. Mice injected with 410.4-IL-12 cells survived significantly ($P < .001$) longer than mice injected with an equivalent number of non IL-12-secreting 410.4 cells (92 ± 14 days and 56 ± 7 days respectively). Eventually, both groups of mice died from progressive tumor growth.

One possibility explanation for the delayed appearance and slower growth of 410.4-IL-12 cells in BALB/c mice is that expression of

neomycin phosphotransferase, a xenoantigen, by the cells was responsible for the difference the cells' growth properties. This point was investigated by comparing the survival of BALB/c mice injected with 410.4 cells transduced with pZipNeoSV(X), a retro viral vector that specified neomycin phosphotransferase, but lacked the gene for IL-12, with the survival of mice injected with the IL-12-secreting 410.4 cells. In the experiment, naive mice were injected into the fat pad of the breast with 1 X 10^6 410.4-IL-12 cells, or with an equivalent number of neomycin-resistant 410.4 cells transduced with pZipNeoSV(X). As an additional control, other naive mice were injected with the same number of non transduced 410.4 cells. The results indicated that the median survival of mice injected with cells transduced with pZipNeoSV(X), 65 ± 15 days, was significantly ($P < .001$) less than that of mice injected with an equivalent number of 410.4 cells modified for IL-12-secretion (94 ± 19) days. It was not significantly different than that of mice injected with unmodified 410.4 cells alone (60 ± 11) days ($p = .1$).

Conceivably, differences in rate of proliferation of 410.4, 410.4 cells transduced with pZipNeoSV(X) and 410.4-IL-12 cells accounted for the differences in the rates of tumor growth, and survival of mice injected with 410.4-IL-12 cells. This point was investigated by measuring in vitro the generation times of the three cell types. The results indicated that the doubling times of the cells, approximately 36 hrs., were not significantly different from each other.

3. Progressively growing neoplasms formed in BALB/c nude mice injected with 410.4-IL-12 cells.

The results of the previous experiments indicated that BALB/c mice injected with 410.4-IL-12 cells survived significantly longer than BALB/c mice injected with an equivalent number of 410.4 cells. The prolonged survival of mice injected with 410.4-IL-12 cells may have been an indication the cells' immunogenic properties were enhanced by IL-12-secretion.

To investigate this question, BALB/c nude mice were injected with 410.4-IL-12 or 410.4 cells. The time to first appearance of tumor, and survival, were compared. As indicated (Figure 2), unlike immunocompetent BALB/c mice, there were no significant differences in the time to first appearance of tumor, and survival of BALB/c nude mice injected with 410.4-IL-12 cells, or 410.4 cells. The median survival time, 46 ± 4 days for mice injected with 410.4-IL-12 cells, was not significantly different that the median survival time of mice injected with 410.4 cells, (43 ± 9 days; P = .32). The results were consistent with an enhancement of the immunogenic properties of 410.4 cells by modification for IL-12-secretion.

4. Progressively growing neoplasms developed in BALB/c mice injected with a mixture of 410.4-IL-12 cells and 410.4 breast cancer cells.

Differences in the time to first appearance of tumor, and survival, of BALB/c mice injected with 410.4-IL-12 cells suggested that the cells' immunogenic properties were enhanced by IL-12-secretion. This point was investigated further by comparing time to first appearance of tumor, and survival, of BALB/c mice injected into the fat pad of the breast with a mixture of 410.4 cells and 410.4-IL-12 cells with the time to first appearance of tumor, and survival, of BALB/c mice injected with an equivalent number of 410.4 cells alone. The number of 410.4 cells was the same in both instances. Conceivably, the presence of 410.4-IL-12 cells would delay the growth of 410.4 cells. As indicated (Figure 3B), the time to first appearance of tumor in mice injected with the mixture of 410.4-IL-12 cells and 410.4 cells was not significantly different than the time to first appearance of tumor in mice injected with 410.4 cells alone (Figure 3A). Furthermore, the median survival time of mice injected with the mixture of 410.4-IL-12 cells and 410.4 cells was not significantly different than the median survival time of mice injected with 410.4 cells alone (MST = 42 ± 13 days and 60 ± 5 days respectively, $p = .06$). As previously, the time to first appearance of tumor in mice injected with 410.4-IL-12 cells alone was significantly ($P < .01$) delayed, relative to that of mice in either of the other groups (Figure 3C). Thus, if 410.4-IL-12 cells stimulated an immune response toward 410.4 cells, the response was insufficient to inhibit the growth of unmodified 410.4 cells.

One possible explanation for the failure of 410.4-IL-12 cells to control tumor growth is that 410.4 cells secrete immunosuppressive

factors such as prostaglandin E2 that inhibit cell mediated immune responses (19-21). This point was investigated by adding culture supernatants from 410.4 cells to a mixed cell culture consisting of spleen cells from naive BALB/c mice (H-2^d) and allogeneic LM fibroblasts (H-2^k). The allogeneic cells were mitomycin-C-treated (50 ug/ml; 30 min., 37°) before they were added to the spleen cell-suspension. Spleen cell proliferation was determined by the adding ^{3}H-thymidine to the mixed cell cultures for the last 18 hrs. of the incubation, followed by measuring the incorporation of ^{3}H-thymidine by the proliferating cells. The results indicated that the addition of culture supernatants of 410.4 cells reduced the proliferative response by approximately five fold (2142 ± 71 cpm in the presence of supernatant vs. 10664 ± 313 in the absence of supernatant.), consistent with the secretion of immunosuppressive factors by the breast cancer cells.

5. Partial immunity toward 410.4 cells was generated in BALB/c mice injected with X-ray-inactivated 410.4-IL-12 cells.

The delayed first appearance of tumor and prolonged survival of BALB/c mice injected with 410.4-IL-12 cells suggested that the weakly immunogenic properties of 410.4 cells had been enhanced by IL-12-secretion, but that the enhanced immunogenic properties were insufficient to control tumor growth. Inactivation of the cells by X-irradiation might preserve the cells' immunogenic properties as it prevented the cells from dividing. This point was investigated by

immunizing naive BALB/c mice with X-irradiated 410.4-IL-12 cells and then challenging the mice by an injection of 410.4 cells. In the experiment, the mice were injected s.c. three times with 1 X 10^6 X-irradiated (5000 rads from a ^{60}Co source) 410.4-IL-12 cells at monthly intervals. Thirty days after the last injection, the mice were injected into the fat pad of the breast with 1 X 10^6 410.4 cells. As controls, naive BALB/c mice were injected according to the same schedule with equivalent numbers of X-irradiated 410.4 cells, or with X-irradiated 410.4-SV-X cells. The results (Figure 4) indicated that one hundred percent of the mice injected with the irradiated 410.4-IL-12 cells, followed by the challenging injection of 410.4 cells, survived indefinitely, more than 90 days. The mice appeared to have rejected the breast cancer cells. Under similar conditions, all of the mice injected with X-irradiated 410.4 cells, or with 410.4-SV-X cells, followed by the challenging injection of 410.4 cells, developed tumors and died from progressive tumor-growth.

6. Immunizations with X-irradiated 410.4-IL-12 cells induced long-term immunity to 410.4 cells in BALB/c mice.

BALB/c mice injected with irradiated 410.4-IL-12 cells were resistant to a challenging injection of unmodified 410.4 cells. To determine if mice that survived the first challenging injection were resistant to a second injection of 410.4 cells, the surviving mice were injected a second time with 410.4 cells 100 days after the first injection. As indicated (Figure 5), BALB/c mice that survived the first injection were resistant to 410.4 cells. They survived

significantly ($P < .001$) longer than naive mice injected with 410.4 cells alone.

DISCUSSION

Under ordinary circumstances, mice injected with viable, syngeneic tumor cells develop progressively growing neoplasms that lead, eventually, to the animals' death. The mice exhibit no resistance to the growth of the malignant cells. Survival of mice injected with the viable cells is inversely related to the number of cells injected. The tumorigenic properties of the cells can be inhibited, however, if the cells are genetically modified to secrete immune augmenting cytokines such as IL-2, GM-CSF or IL-12 (12). Normal cells appear to be unaffected. This seminal finding was an indication that tumor cells expressed weakly immunogenic, tumor associated antigens. They were the products of mutated or dysregulated genes in the tumor cells that differed from the homologous genes in nonneoplastic cells of the tumor-bearing host (24,25). Under appropriate circumstances, such as in the microenvironment of the immune-augmenting cytokines, the TAAs can become immunogenic and become the targets of immune-mediated attack. The clinical significance of these findings is that the experimental animals that rejected the cytokine-secreting tumor cells developed systemic, cell mediated immunity directed toward both the cytokine-secreting as well as non secreting tumor cells (12), pointing toward the important potential of this form of cancer therapy.

Here, we investigated the immunotherapeutic properties of IL-12-secreting 410.4 breast adenocarcinoma cells in BALB/c mice,

syngeneic with the tumor. IL-12 is a pleotropic, heterodimeric immune-augmenting cytokine (26-29) that stimulates both natural killer (NK) and T lymphocytes to produce interferon-gamma and tumor necrosis factor-alpha. IL-12 also promotes the development of Th1 $CD4^+$ cells, and enhances the activity of tumor infiltrating lymphocytes (30,31). Our results indicated, however, that in spite of these immune enhancing effects, IL-12-secretion by the breast cancer cells failed to eliminate the cells' tumorigenic properties. Progressively growing neoplasms formed in mice injected with 410.4-IL-12 cells. However, IL-12 appeared to augment the cells' immunogenic properties since tumor growth was delayed in immunocompetent BALB/c mice injected with the IL-12-secreting cancer cells. In contrast, the rate of tumor-formation in BALB/c nude mice injected with 410.4-IL-12 cells or unmodified 410.4 cells were essentially the same. The unimpaired growth of 410.4-IL-12 cells in nude mice suggested that NK cells were insufficient to control tumor growth (14).

Immunocompetent BALB/c mice immunized with X-irradiated 410.4-IL-12 cells were resistant to a subsequent challenging injection of unmodified 410.4 cells. Under similar conditions, immunizations with X-irradiated (non IL-12-secreting) 410.4 cells failed to induce tumor-immunity. We concluded that IL-12-secretion by 410.4 cells augmented the cells' immunogenic properties, but that the increased immunogenicity of the cells was insufficient to control tumor formation. This may be a unique finding as Kundu et al. (32) reported

that 410.4 cells modified to secrete IL-10 failed to form tumors in syngeneic mice.

The potential importance of these findings to breast cancer patients is indicated by reports indicating that spontaneous breast neoplasms arising in patients, like other types of cancer, are potentially immunogenic. They form tumor associated antigens that can be recognized by cytotoxic T lymphocytes. Cytotoxic T lymphocytes were detected in breast cancer patients with specificity for ErbB-2 (33,34), mutant p53 (35) MAGE-1 (36), BAGE (37) and the protein core of mucin expressed in an altered form by breast cancer cells (38-40). These may be only several of an array of breast cancer associated antigens that arise from altered genes in the malignant cells. Genetic instability is a common characteristic of breast cancer and other types of neoplastic cells (41-44).

These data indicate that the immunogenic properties of a cell line derived from an adenocarcinoma of the breast can be enhanced if the cells are modified to secrete IL-12. They point toward the immunotherapeutic potential of X-irradiated breast cancer cells modified for cytokine-secretion. The finding may be of importance in the clinical care of breast cancer patients.

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References

1. Fearon ER, Pardoll DM, Itaya T, Golumbek PT, and Levitsky HI. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell* 1990;60:397-403.

2. Cavallo F, Pierro FD, Giovarelli M, et al. Protective and curative potential of vaccination with interleukin-2-gene-transfected cells from a spontaneous mouse mammary adenocarcinoma. *Cancer Res.* 1993;53:5067-5070.

3. Connor J, Bannerji R, Saito S, Heston W, Fair W, Gilboa, E. Regression of bladder tumors in mice treated with interleukin 2 gene-modified tumor cells. *J. Exp. Med.* 1993;177:1127-1134.

4. Chakravarty PK, Fuji H , Abu-hadid MM, Hsu S-C, Sood AK. Tumorigenicity of interleukin-2 (IL-2)-cDNA-transfected L1210 lymphoma and its in vivo variants is modulated by changes in IL-2 expression: potential therapeutic implications. *Cancer Immunol. Immunother.* 1992;35:347-354.

5. Russell SJ, Eccles SA, Flemming CL, Johnson CA, Collins MKL. Decreased tumorigenicity of a transplantable sarcoma following transfer and expression of IL-2 cDNA. *Int. J. Cancer* 1991;47:244-251.

6. Hock H, Dorsch M, Kunzendorf U, Qin Z, Diamanstein T, Blankenstein T. Mechanism of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon g. *Proc. Natl. Acad. Sci. USA* 1993;90:2774-78.

7. Golumbek PT, Lazenby AJ, Levitsky HI, Jaffee LM, Karasuyama H, Baker M, Pardoll DM. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science* 1991;254:713-716.

8. Columbo MP, Ferrari G, Stoppacciaro A, Parenza M, Rodolfo M, Mavilio F, Parmiani G. Granulocyte colony-stimulating factor gene transfer suppresses tumorigenicity of a murine adenocarcinoma *in vivo*. *J. Exp. Med.* 1991;173:889-897.

9. Dranoff G, Jaffee E, Lazenby A, et al. Vaccination with irradiated tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA* 1993;90:3539-3543.

10. Gansbacher B, Bannerji R, Daniels B, Zier K, Cronin K, Gilboa E. Retroviral vector-mediated g-interferon gene transfer into tumor cells generates potent and long lasting antitumor immunity. *Cancer Res.* 1990;50:7820-25.

11. Nayak SK; McCallister T; Han LJ; Gangavalli R; Barber J; Dillman RO Transduction of human renal carcinoma cells with human gamma-interferon gene via retroviral vector. Cancer Gene Ther 1996;3:143-50.

12. Pardoll DM Paracrine cytokine adjuvants in cancer therapy. *Annu. Rev. Immunol.* 1995;13: 399-415.

13. Zitvogel L, Tahara H, Robbins PD, Storkus WJ, Clarke MR, Nalesnik MA, Lotze MT Cancer immunotherapy of established tumors with IL-12. *J Immunol.* 1995;155:1393-1403.

14. Brunda MJ, Luistro L, Warrier RR, et al. Antitumor and antimetastatic activity of interleukin-12 against murine tumors. *J. Exp Med.* 178:1223-1230,1993.

15. Tahara H, Zeh HJ, Storkus WJ, et al. Fibroblasts genetically engineered to secrete interleukin 12 can suppress tumor growth and induce antitumor immunity to a murine melanoma in vivo. *Cancer Res.* 1994;54:182-89.

16. Rodolfo M, Zilocchi C, Melani C, Cappetti B, Arioli I, Parmiani G, Colombo MP. Immunotheray of experimental metastses by vaccination with interleukin gene-transduced adenocarcinoma cells sharing tumor associated antigens. *J Immunol.* 1996:157,5536-5542.

17. Brunda MJ, Luistro L, Henderzak JA, et al. Role of interferon-g in mediating the anti tumor efficacy of IL-12. *J. Immunotherapy* 1995;17:71-77.

18. Miller FR, Miller BE, Heppner GH. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: Heterogeneity in phenotype stability. *Invasion Met.* 1983;3:22-31.

19. Fulton AM, Heppner GH. Relationships of prostaglandin and natural killer sensitivity to metastatic potential in murine mammary adenocarcinomas. *Cancer Res.* 1985;45:4779-84.

20. van der Pouw Kraan TCTM, Boeije LCM, Smeenk RJT, Wijdenes J and Aarden LA Prostaglandin-E2 is a potent inhibitor of human interleukin-12 production. *J Exp Med* 1995;181:775-79.

21. Rieser C, Bock G, Klocker H, Bartsch G and Thurnher M. Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin-12 production. *J. Exp. Med.* 1997;186:1603-08.

22. Tahara M, Lotze MT. Anti tumor effects of IL-12: Applications for the immunotherapy and gene therapy of cancer. *Gene Therapy* 1995;2:96-106.

23. Tapscott SJ, Miller AD, Olson JM et al. Gene therapy of rat 9L gliosarcoma tumors by transduction with selectable genes does not require drug selection. *Proc Natl Acad Sci* USA 1994;91:8185-8189.

24. Boon T, Cerottini J-C, van den Eynde et al. Tumor antigens recognized by T lymphocytes. *Ann Rev Immunol.* 1994;12:337-65.

25. Boon T. Toward a genetic analysis of tumor rejection antigens. *Advances in Cancer Research* 1992;58:177-210.

26. Germann T, Rude E. Interleukin-12. *Int Arch Allery Immunol* 1995:103-112.

27. Schoenhaut DS, Chua AO, Wolitzky AG, et al. Cloning and expression of murine IL-12. *J Immunol.* 1992;148:3433-40.

28. Gubler U, Chua AO, Schoenhaut DS, Dwyer CM, Gately MK. Coexpression of two distinct genes is required to generate secreted

biactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci USA* 1991 88:4143-49.

29. Wolf SF, Temple PA, Kobayashi M, Trinchieri G, Perussia B. Cloning of cDNA for NKSF, a heterodimeric cytokine with multiple biologic effects on T and NK cells. *J Immunol.* 146: 3074-79, 1991.

30. Kennedy MK, Picha KS, Shanebeck KD, et al. IL-12 regulates the proliferation of Th1, but not Th2 or Th0 clones. *Eur J Immunol.* 1994;24:2271-277.

31. Hendrzak JA, Brunda MJ Antitumor and antimetastatic activity of IL-12. *Curr Top Microbiol Immunol.* 1996;213(pt3):65-83.

32. Kundu N, Beaty TL, Jackson MJ, Fulton AM. Antimetastatic and antitumor activities of IL-10 in a mouse model of breast cancer. *J Natl Cancer Inst* 1996;88:536-541.

33. Disis L, Calenoff E, McLaughlin G, et al. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breastcancer. *Cancer Res* 1994;54:16-20.

34. Peoples GE, Goedegeburre PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *Proc Natl Acad Sci* USA 1995;92:432-36.

35. Schlichtholz B, Legros Y, Gillet D, Gaillard C, Marty M, lane D, Calvo F and Soussi T. The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hotspot. *Cancer Res.* 1992;52:6380-84.

36. Toso JF, Oei C,Oshidari F, Tartaglia J, Paoletti E, Lyerly HK, Talib S, Weinhold KJ. MAGE-1-specific precurser cytotoxic T lymphocytes present among tumor-infiltrating lymphocytes from a patient with breast cancer: characterization and antigen-specific activation. *Cancer Res* 1996;56:16-20.

37. Jerome KR, Barnd DL, Bendt , et al. Cytotoxic T-lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on protein core of a mucin molecule preferentially expressed by malignant cells. *Cancer Res.* 1991;51:2908-2916.

38. Fuije T, Mori M, Ueo H, Sugimachi K, Akioshi T. Expression of MAGE and BAGE genes in Japanese breast cancers. *Ann Oncol* 1997;8:369-72.

39. Domenenech N, Henderson RA, Finn OJ. Identification of an HLA-A11-restricted epitope from the tandem repeat domain of the epithelial tumor antigen mucin. *J Immunol* 1995;155:4766-74.

40. Jerome KR, Domenech N, Finn OJ. Tumor-specific cytotoxic T lymphocyte clones frompatients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cell transfected with polymorphic epithelial mucin cDNA. *J Immunol.* 1992;151:1644-59.

41. Thibodeau S, Bren G and Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993;260:816-19.

42. Gonzalez-Zulueta M, Ruppert JM, Tokino K, Tsai YC, Spruck III CH, Miyao N, Nichols PW, Hermann GG, Horn T, Steven K, Summerhayes IC, Sidransky D and Jones PA. Microsatellite instability in bladder cancer. *Cancer Res* 1993;53:5620-23.

43. Risinger JI, Berchuck A, Kohler MF, Watson P, Lynch HT, Boyd J. Genetic instability of microsatellites in endometrial carcinoma. *Cancer Res* 1993;53:5100-03.

44. Peltomaki P, Lothe R, Aaltonen LA, et al. Microsatellite instability is associated with tumors that characterize the HNPCC syndrome. *Cancer Res* 1993;53:5853-55.

Legends

Figure 1. Tumor growth in BALB/c mice injected into the fat pad of the breast with 410.4-IL-12 Cells.

Legend: Naive BALB/c mice were injected into the fat pad of the breast with 1 x 10^6 410.4-IL-12 cells, or, for comparison, with an equivalent number of unmodified 410.4 cells. Each cell-type was suspended in 200 ul of growth medium for the injection. Two dimensional tumor measurements were performed and the volume of the tumor was calculated as described in the Materials and Methods section. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

Figure 2. Tumor growth in BALB/c nude mice injected into the breast with 410.4-IL-12 or 410.4 cells.

Legend: Naive BALB/c nude mice were injected into the fat pad of the breast with 1 x 10^6 410.4-IL-12 cells, or, for comparison, with an equivalent number of unmodified 410.4 cells. Each cell-type was suspended in 200 ul of growth medium for the injection. Two

dimensional tumor measurements were performed and the volume of the tumor was calculated as described in the Materials and Methods section. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

Figure 3. Tumor growth in BALB/c mice injected into the breast with a mixture of 410.4-IL-12 and 410.4 cells

Legend: Tumor growth was measured in naive BALB/c mice injected into the fat pad of the breast with a mixture of 1 X 10^6 410.4-IL-12 cells and 1 X 10^6 410.4 cells in a total volume of 200 ul growth medium [B]. For comparison, naive mice were injected with 1 X 10^6 410.4 cells [A] or 1 X 10^6 410.4-IL-12 cells in 200 ul growth medium alone [C]. Two dimensional tumor measurements were performed and the volume of the tumor was calculated as described in the Materials and Methods section. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

Figure 4. Survival of BALB/c mice injected with irradiated 410.4-IL-12 cells, followed by a challenging injection of 410.4 cells.

Legend: BALB/c mice were injected s.c. with 1 x 10^6 X-irradiated (5000 rads from a ^{60}Co-source) 410.4, 410.4-SV-X, or 410.4-IL-12 cells. The mice received three injections at intervals of 30 days. Thirty days after the last injection, the mice were injected into the fat pad of the breast with 1 x 10^6 410.4 cells suspended in a volume of 200 ul of growth medium.

□ = mice injected with irradiated 410.4 cells, followed by 410.4 cells

○ = mice injected with irradiated 410.4-SV-X cells, followed by 410.4 cells

■ = mice injected with irradiated 410.4-IL-12 cells, followed by 410.4 cells

Figure 5. Tumor growth in surviving BALB/c mice reinjected with 410.4 cells.

Legend: BALB/c mice injected previously with irradiated 410.4-IL-12 cells, followed by an injection of 410.4 cells, were injected into the fat pad of the breast a second time with 1 x 10^6 viable 410.4 cells suspended in 200 ul of growth medium. Naive BALB/c mice were injected into the fat pad of the breast with an equivalent number of 410.4 cells suspended in 200 ul of growth medium.

□ = naive BALB/c mice injected with 410.4 cells.

■ = surviving BALB/c mice injected with 410.4 cells.

The inset represents tumor growth in individual mice. Mice with tumor growth were naive mice injected with 410.4 cells. Mice without tumor growth were immunized mice surviving a prior injection of 410.4 cells, re-injected with 410.4 cells.

Figure 2

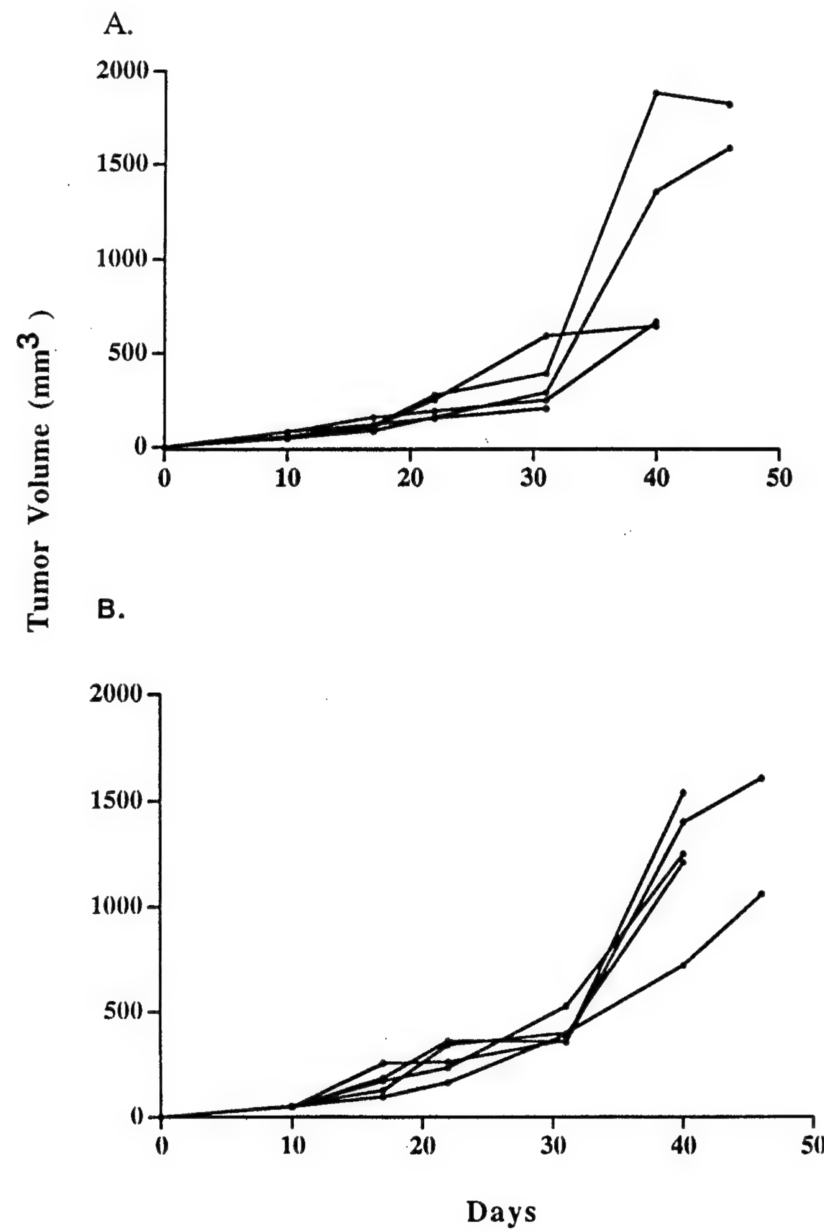
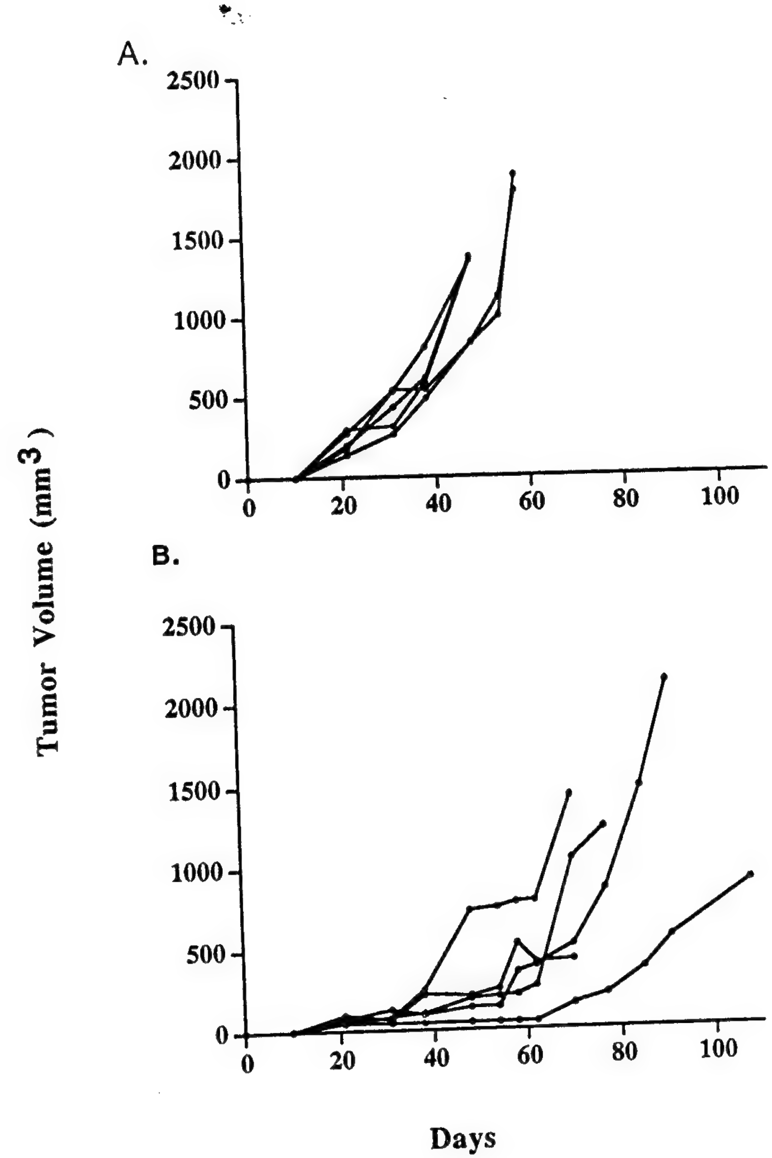


Figure 1



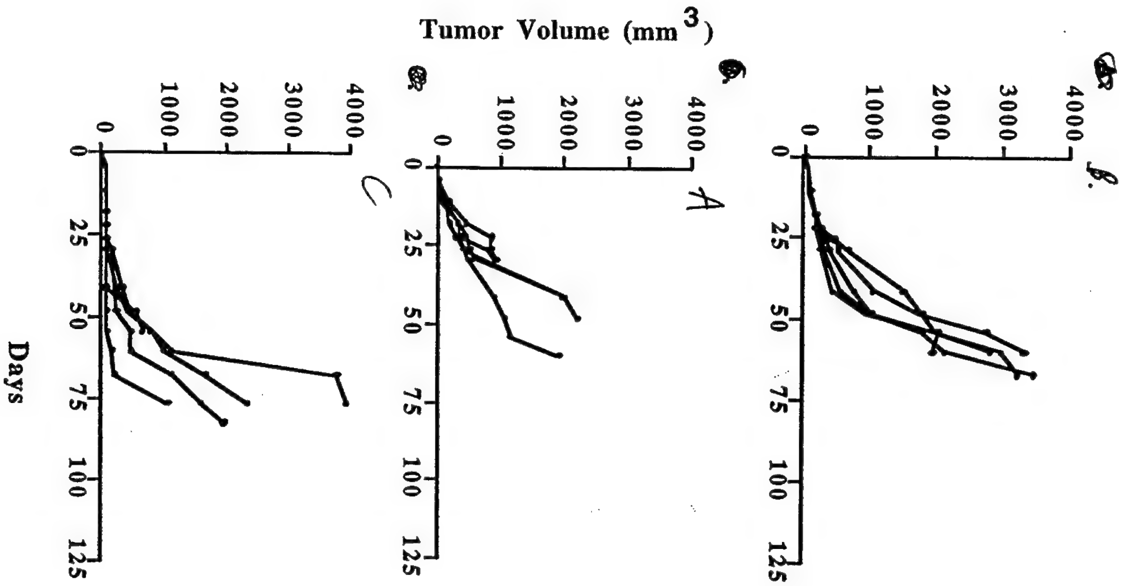


Figure 3

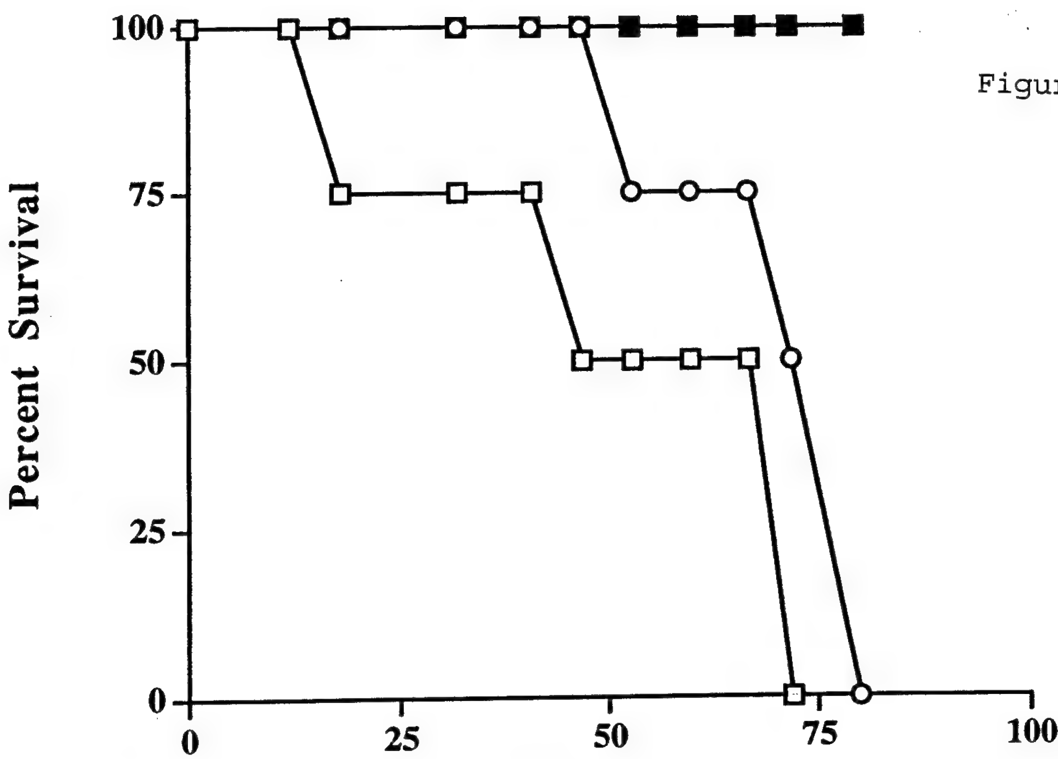
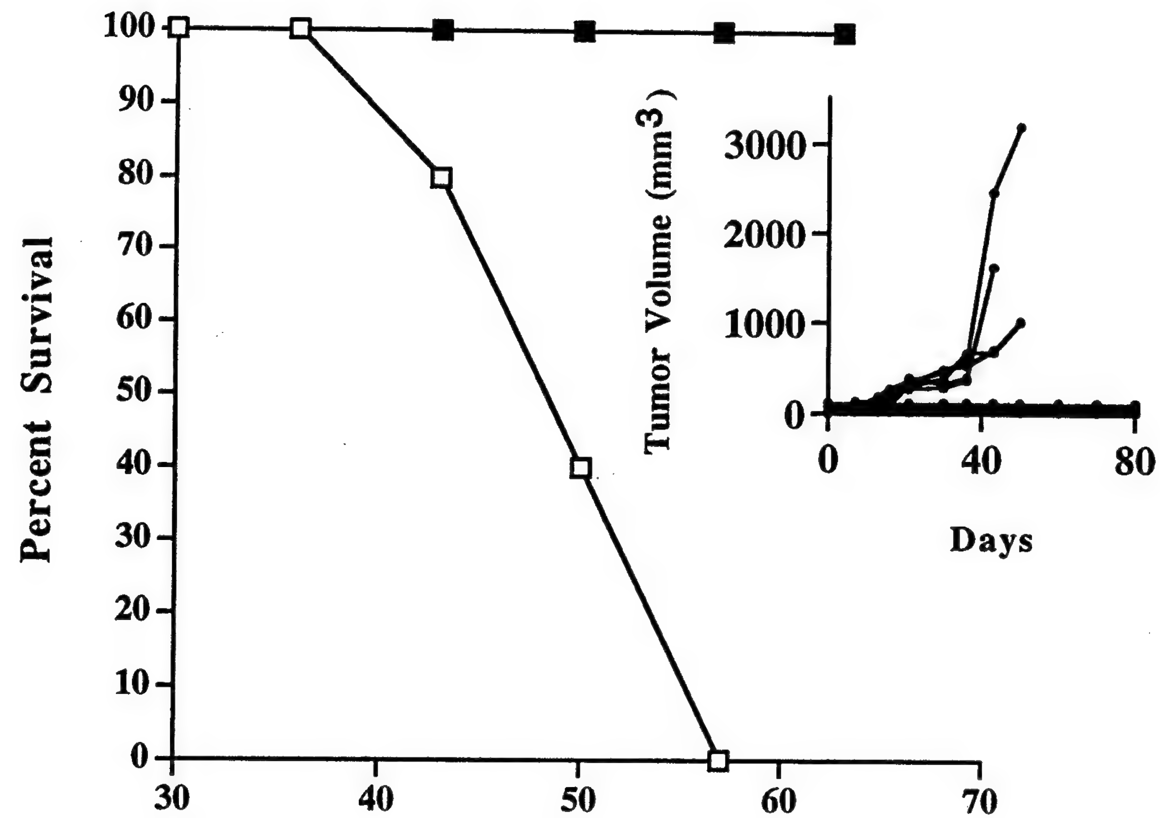


Figure 4

Figure 5



Percent Survival
100
90
80
70
60
50
40
30
20
10
0
30
40
50
60
70
Tumor Volume (mm3)
3000
2000
1000
0
0
40
80
Days

Treatment of Breast Cancer with Fibroblasts Transfected with DNA from Breast Cancer Cells

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Abstract:

Background: The objective of this investigation was to determine if mice with breast cancer could be successfully treated by immunization with fibroblasts transfected with DNA from breast cancer cells. The immunogenic properties of breast cancer-associated antigens (TAAs), the products of mutant or dysregulated genes in the malignant cells, can be enhanced if they are expressed by highly immunogenic cells. Methods: We transfected LM mouse fibroblasts (H-2^k) modified beforehand to secrete interleukin-2 (IL-2) with genomic DNA from a breast adenocarcinoma that arose spontaneously in a C3H/He mouse (H-2^k). To increase their immunogenic properties, the fibroblasts were also modified to express allogeneic MHC-determinants (H-$2K^b$). Afterward, the modified cells were co transfected with DNA from the spontaneous breast neoplasm, along with a plasmid (pHyg) conferring resistance to hygromycin, used for selection. Pooled colonies of hygromycin-resistant cells were then tested in C3H/He mice for their immunotherapeutic properties against the growth of the breast neoplasm. Results: Tumor-bearing mice immunized with the transfected cells survived significantly ($p < .01$) longer than mice in various control groups. Similar beneficial effects

were seen in C57BL/6J mice injected with a syngeneic breast carcinoma cell line (E0771) and modified fibroblasts transfected with DNA from E0771 cells. The immunity was mediated by $CD8^+$ T cells since immunized mice depleted of $CD8^+$ cells failed to resist tumor growth. Conclusions: A vaccine effective in the treatment of breast cancer was prepared by transfection of breast cancer DNA into a highly immunogenic fibroblast cell line.

Introduction

Cancer cells form weakly immunogenic, tumor associated antigens (TAAs) (1-4) that can be recognized by cytotoxic T lymphocytes (CTLs). The TAAs are the products of dysregulated, or mutant genes in the neoplastic cells that differ from the homologous genes in non neoplastic cells of the same individual. Like other neoplasms, breast cancer cells form TAAs. The products of genes specifying HER-2/neu (5) MAGE-1 (6), BAGE (7), and MUC-1 (8-10) expressed by breast cancer cells have been identified as targets of CTLs. These may be only several representations of an undefined, and possibly large number of tumor antigens expressed by the malignant cells. Genetic instability is a characteristic phenotype of breast cancer and other types of malignant cells (11-15).

Under appropriate circumstances, tumor-specific cellular immune responses can be induced against TAAs expressed by neoplastic cells. The immune responses can be of sufficient magnitude to prolong the lives of tumor-bearing animals (16-20), and patients (21,22). Genetic modification of tumor cells to secrete cytokines has been used as one means of augmenting the immunogenic properties of the malignant cells.

Expression-competent genes for IL-2 (23-27), IL-4 (28), IL-6 (29), IL-7 (30), IL-12 (31), TNF-α (32, 33), IFN-α and γ (34, 35), GM-CSF (36, 37) among others (38) have been introduced into neoplastic cells for this purpose. Immunizations with the cytokine-secreting, tumor cells resulted in cellular immune responses that were directed toward the malignant, but not the non malignant cells of the tumor-bearing host. Analogous tumor-specific responses were induced if the neoplastic cells used for the immunizations were modified to express syngeneic or allogeneic MHC determinants (39-42), or to express co stimulatory molecules such as B7, required for activation of immune effector cells (43). However, the direct modification of cells from a primary neoplasm requires the establishment of a tumor cell line. This can be technically challenging, and may not always succeed. This is especially the case for breast cancer. Breast cancer cell lines are notoriously difficult to establish from primary breast neoplasms.

In other instances, defined tumor antigens or unfractionated tumor peptides have been used for tumor immunotherapy. However, few defined tumor antigens have been identified and cloned, and immunization with unfractionated tumor peptides requires large amounts of tumor if

multiple immunizations are to be carried-out. Sufficient quantities of tumor tissue may not be available if patients are in clinical remission.

Here, we tested an alternative approach. Classic studies indicated that transfection of DNA from one cell type can stably alter both the genotype and the phenotype of cells that take-up the exogenous DNA. Wigler et al. (44), for example, reported stable integration of the gene for adenine phosphoribosyltransferase into mouse cells deficient in the enzyme by transfection of high molecular weight genomic DNA from adenine phosphoribosyltransferase-positive mouse cells. A similar approach was used to convert thymidine kinase-deficient mouse cells to cells that expressed thymidine-kinase by transfer of genomic DNA from a variety of thymidine-kinase-positive tissues and cultured cells (45). In an analogous manner, Mendersohn et al. (46) reported that polio virus receptor-negative cells could be converted to cells that expressed the receptor by transfection of genomic DNA from receptor-positive cells. The products of single genes specifying the enzymes, or membrane associated determinants were expressed by subpopulations of the transfected cells.

We prepared a cellular vaccine that was capable of prolonging the survival of mice with breast cancer by transfecting DNA from the breast cancer cells into a highly immunogenic cell line. We reasoned that genes specifying numerous, undefined, weakly immunogenic TAAs would be expressed in a highly immunogenic form by the transfected cells, and that immunizations with the transfected cells might result in an immune response directed toward the breast cancer cells. We used two types of breast tumors, with analogous results. DNA from an adenocarcinoma of the breast that formed spontaneously in a C3H/He mouse was used to transfect a mouse fibroblast cell line. A plasmid (pHyg) specifying resistance to hygromycin was included to allow selection of cells that had taken-up the exogenous DNA. The antibiotic-resistant, transfected cells were then used to treat mice with breast cancer. The results indicated that mice immunized with the transfected fibroblasts developed generalized, cell-mediated immunity toward the breast cancer cells. The treated animals survived significantly longer than mice in various control groups, including mice with breast cancer treated by immunization with non DNA-transfected fibroblasts. Similar results were obtained for mice bearing a mammary adenocarcinoma cell line (E0771) of C57BL/6J mouse origin treated with fibroblasts transfected

with DNA from EO771 cells. The immunity was mediated by $CD8^+$ T lymphocytes.

The augmented resistance to breast cancer in mice treated with fibroblasts transfected with breast cancer DNA points toward an analogous form of therapy for breast cancer patients.

Materials and Methods

Cell lines and experimental animals. Eight to 10 week old pathogen-free C3H/HeJ mice (H-2^k) and 8 to 10 week old pathogen-free C57BL/6J mice (H-2^b) were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained in the animal care facilities of the University of Illinois, according to NIH Guidelines for the Care and Use of Laboratory Animals. They were 8 to 12 weeks old when used in the experiments. EO771 cells, a mammary adenocarcinoma cell line derived from a C57BL/6J mouse, were from the Tumor Repository of the Division of Cancer Treatment, Diagnosis and Centers of the National Cancer Institute (Frederick, MD). SB-1 cells were a breast adenocarcinoma that formed spontaneously in a C3H/HeJ mouse. B16 cells, a melanoma cell line originating in a C57BL/6J mouse, were from I. Fidler (MD Anderson, Houston, TX). EO771 cells were maintained by serial passage in histocompatible C57BL/6J mice. B16 cells were maintained by serial passage in C57BL/6J mice or at 37°C in a humidified 7% CO_2/air atmosphere in DMEM (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and antibiotics (Gibco BRL) (growth medium). LM cells, a fibroblast cell line of C3H/He mouse origin,

were from the American Type Culture Collection (Rockville, MD). The cells were maintained at 37°C in a humidified 7% CO_2/air atmosphere in growth medium.

Modification of LM mouse fibroblasts for IL-2-secretion. LM fibroblasts were modified for IL-2-secretion by transduction with the retro viral vector pZipNeoSVIL-2 (from M.K.L. Collins, University College, London, England) (LM-IL-2 cells). The vector, packaged in GP+*env* AM12 cells (from A. Bank, Columbia University, New York, NY), included a human IL-2 cDNA and a *neo*^{r} gene, both under control of the Moloney leukemia virus long terminal repeat. The *neo*^{r} gene conferred resistance to the aminoglycoside antibiotic, G418. Virus-containing supernatants of GP+*env* AM12 cells transfected with pZipNeoSVIL-2 were added to LM fibroblasts, followed by overnight incubation at 37°C in growth medium to which polybrene (Sigma; 5 ug/ml, final concentration) had been added. The cells were maintained for 14 days in growth medium containing 400 ug/ml G418 (Gibco BRL). One hundred percent of non transduced LM cells died in the medium supplemented with G418 during this period. Colonies of cells proliferating in the G418-containing growth medium were pooled for later use in the experiments. Every third, fourth and fifth passage,

the transduced cells were cultured in growth medium containing 400 ug/ml G418. IL-2 secretion by LM-IL-2 cells was detected by the capacity of supernatants from the transduced cells to sustain the growth of CTLL-2 cells, an IL-2-dependent cell line (47). Varying dilutions of the filtered culture supernatants (0.2 um nitrocellulose; Gelman, Ann Arbor, MI) were transferred to 96-well plates containing 1 x 10^4 CTLL-2 cells in a final volume of 200 ul of growth medium per well. After incubation for 16 hours, 0.5 uCi ^{3}H-thymidine (Amersham, Arlington Heights, IL) was added to each well for 6 additional hours of incubation. A standard curve was generated by adding varying amounts of recombinant human IL-2 (Gibco BRL) to an equivalent number of CTLL-2 cells. Afterward, the cells were collected onto glass fiber filters (Whittaker M.A. Products, Walkerville, MD) using a PhD multiple harvester (Microbiological Associates, Bethesda, MD). After washing with ethanol (95%), radioactivity in the insoluble fraction was measured in a liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL). One unit of IL-2 resulted in half maximal proliferation of CTLL-2 cells under these conditions.

Modification of LM-IL-2 cells for the expression of H-2K^b class I-determinants. pBR327H-2K^b (Biogen Research Corp., Cambridge, MA), a plasmid encoding MHC H-2K^b (48), was used to modify LM-IL-2 fibroblasts for the expression of H-2K^b-determinants (LM-IL-2K^b cells). Ten ug of pBR327H-2K^b and 1 ug of pBabePuro (from M. K. L. Collins) a plasmid conferring resistance to puromycin (49) were mixed with Lipofectin (Gibco BRL), according to the supplier's instructions, and then added to 1 x 10^6 LM-IL-2 cells in 10 ml of DMEM without FBS. For use as a control, an equivalent number of LM-IL-2 cells was transfected with 1 ug of pBabePuro alone. The cells were incubated for 18 hours at 37°C in a CO_2/air atmosphere, washed with DMEM, followed by the addition of 7 ml of growth medium. After incubation for 48 hours, the cell cultures were divided and re plated in growth medium supplemented with 3 ug/ml puromycin (Sigma; St. Louis, MO) followed by incubation at 37°C for 7 additional days. The surviving colonies were pooled and tested by staining with specific FITC-conjugated antibodies (described, below) for the expression of H-2K^b-determinants. One hundred percent of non-transfected LM-IL-2 cells maintained in growth medium containing puromycin died during the seven day period of incubation.

Immunofluorescent staining and cytofluorometric measurements. Quantitative immunofluorescent staining was used to detect the expression of H-2K^b-determinants by LM-IL-2 cells transfected with pBR327H-2K^b. The measurements were performed in an Epic V flow cytofluorograph (Coulter Electronics, Hialeah, FL) equipped with a multiparameter data-acquisition and display system (MDADS). For the analysis, a single cell suspension was prepared from the monolayer cultures of puromycin-resistant cells with 0.1 mM EDTA in 0.1 M phosphate buffered saline, pH 7.4 (PBS). The cells were washed with PBS containing 0.2% sodium azide and 0.5% FBS. Afterward, FITC-conjugated H-2K^b. H-2K^d or H-2K^k mAbs (Pharmingen, San Diego, CA), or FITC-conjugated IgG_{2a} isotype serum (DAKO, Carpenteria, CA) was added to the cells, followed by incubation at 4^o C for 1 hr. The cells were then washed with PBS containing 0.5% FBS and 0.2% sodium azide. One-parameter fluorescence histograms were generated by analyzing at least 1 X 10^4 cells. Background staining was determined by substituting cells stained with FITC-conjugated mouse IgG_{2a} alone for cells stained with the specific antibodies.

Depletion of mice of CD8$^+$ and CD4$^+$ T cells. Monoclonal antibodies were used to deplete naive C57BL/6J mice of CD8$^+$ and CD4$^+$ T cells. The mice were injected intraperitoneally (i.p.) with the antibody-rich fraction obtained from ascites fluid containing anti CD8 (83-23-5 mouse hybridoma) or from ascites fluid containing anti CD4 (GK1.5 rat hybridoma) (both hybridomas were from Dr. K. Herald, University of Illinois at Chicago). The mice were injected i.p. with 0.3 ml (5 mg) of enriched 83-12-5 antibodies, or i.p. with 0.2 ml (1 mg) of enriched GK1.5 antibodies. Depletion of the relevant subset of T cells was verified by flow cytofluorometric analysis of spleen cell suspensions taken two days after the injection of the enriched ascitic fluid. The depleted conditions were maintained in the remaining mice by injections of equivalent amounts of the appropriate antibodies every five days until the experiments were concluded.

Transfection of LM-IL-2K^b cells with DNA from a breast carcinoma that arose spontaneously in a C3H/He mouse (SB-1), from EO771 breast carcinoma cells or from B16 melanoma cells. Sheared, unfractionated DNA isolated (Qiagen, Chatsworth, CA) from a spontaneous mammary adenocarcinoma (SB-1) taken directly from

a C3H/HeJ mouse, or from EO771 cells taken from a C57BL/6J mouse, or from B16 melanoma cells from in vitro culture, was used to transfect LM-IL-2K^b cells. The method described by Wigler et al., (45) was applied, as modified. Briefly, high molecular weight DNA from each cell type was sheared by three passages through a 25 gauge needle. Afterward, 100 ug of the sheared DNA was mixed with 10 ug pHyg (from L. Lau, University of Illinois, Chicago, IL), a plasmid that encoded the *E. Coli* enzyme hygromycin B phosphotransferase (52), conferring resistance to Hygromycin B. The sheared DNA and pHyg were then mixed with Lipofectin, according to the manufacturer's instructions (Gibco BRL). The DNA/Lipofectin mixture was added to a population of 1 X 10^7 LM-IL-2K^b cells that had been divided into ten 100 mm plastic cell culture plates 24 hours previously. Eighteen hours after addition of the DNA/Lipofectin mixture to the cells, the growth medium was replaced with fresh growth medium. For use as a control, DNA from the tumor cells was omitted and 1 ug of pHyg alone, mixed with Lipofectin, was added to an equivalent number of LM-IL-2K^b cells. The same protocol was followed to transfect LM-IL-2 cells (not transduced with pBR327H-2K^b) with DNA from SB-1 cells. In each instance, the cells were maintained for 14 days in growth medium containing 600 ug/ml hygromycin B (Boehringer Mannheim,

Indianapolis, IN). One hundred percent of LM-IL-2K^b or LM-IL-2 cells transfected with tumor-DNA alone maintained in the hygromycin-growth medium died within this period. The surviving colonies (at least 2.5 X 10^4) of LM-IL-2K^b or of LM-IL-2 cells transfected with pHyg and DNA from the tumor cells, or with pHyg alone (LM-IL-2K^b cells), were pooled and used in the experiments.

Results

Modification of LM mouse Fibroblasts for the Secretion of IL-2.

A replication-defective retroviral vector, pZipNeoSVIL-2, was used to modify LM fibroblasts (H-2^k) for the secretion of IL-2. The vector specified the gene for human IL-2, along with a gene (neo^r) that conferred resistance to the neomycin analog, G418. After selection in growth medium containing sufficient quantities of G418 to kill one hundred percent of non transduced cells, the surviving colonies were pooled and maintained as a cell line. Analysis of the culture supernatants indicated that 1 X 10^6 retrovirally transduced cells formed 150 U IL-2/10^6 cells/48 hours, as determined by the capacity of the supernatants

to sustain the growth of IL-2-dependent CTLL-2 cells. IL-2-secreting cells modified to express H-$2K^b$-determinants (LM-IL-$2K^b$) and IL-2-secreting cells transfected with tumor DNA (described, below) formed equivalent quantities of IL-2. The culture supernatants of LM cells transduced with the IL-2-negative vector (pZipNeoSV(X), or of non transduced LM cells failed to form detectable quantities of IL-2. Every third, fourth and fifth passage, the IL-2-secreting cells were placed in medium containing 400 ug/ml G418. Under these conditions, similar quantities of IL-2 were detected in the culture supernatants of cells transduced with pZipNeoSVIL-2 for more than 6 months of continuous culture (these data are not presented).

Modification of LM-IL-2 cells for the Expression of MHC class I H-$2K^b$ Determinants.

A plasmid, pBR327H-$2K^b$, was used to modify LM-IL-2 cells for the expression of H-$2K^b$ determinants. LM-IL-2 cells were co transfected with pBR327H-$2K^b$ DNA along with pBabePuro DNA, used for selection. (A 10:1 ratio of pBR327H-$2K^b$ DNA to pBabePuro DNA was used to increase the likelihood that cells that incorporated pBabePuro DNA took up

$pBR327H\text{-}2K^b$ DNA as well.) After selection in growth medium containing sufficient quantities of puromycin to kill the non transduced cells, the surviving colonies were pooled and the cell number was expanded in vitro.

The expression of $H\text{-}2K^b$-determinants by the modified cells was measured by quantitative immunofluorescent staining, using FITC-labeled mAbs for mouse $H\text{-}2K^b$-determinants. As controls, aliquots of the puromycin-resistant cell-suspension were incubated with FITC-labeled IgG_{2a} isotype serum, or with FITC-labeled mAbs for $H\text{-}2K^d$-determinants. As an additional control, the cells were incubated with FITC-labeled $H\text{-}2K^K$ mAbs (LM cells are of C3H/He mouse origin). The mean fluorescent index (MFI) of the puromycin-resistant LM-IL-2 cells stained with FITC-conjugated $H\text{-}2K^b$ or FITC-conjugated $H\text{-}2K^k$ mAbs (xx and xx respectively) was significantly ($p < .001$) higher than that of cells stained with FITC-conjugated $H\text{-}2K^d$ mAbs (Figure 1). The MFI of cells stained with FITC-conjugated $H\text{-}2K^d$ mAbs was approximately the same as that of cells stained with FITC-conjugated IgG_{2a} isotype serum. The expression of $H\text{-}2K^b$-determinants was a stable property of the transfected cells. The cells stained with equivalent intensity with FITC-conjugated $H\text{-}2K^b$ mAbs after three months of continuous culture (these data are not presented).

Survival of C57BL/6J Mice Injected with EO771 Breast Cancer Cells and LM-IL-2K^b Cells Transfected with DNA from EO771 Cells (LM-IL-2K^b/EO771).

C57BL/6J mice are highly susceptible to the growth of EO771 cells, a syngeneic breast cancer cell line. One hundred percent of mice injected with EO771 cells die from progressive tumor-growth.

The effect of immunization with LM-IL-2K^b/EO771 cells on the growth of EO771 cells in C57BL/6J mice was determined by injecting naive mice into the fat pad of the breast with a mixture of EO771 cells and LM-IL-2K^b/EO771 cells, as described in the Legend to Figure 2. At the same time, the mice received an intraperitoneal (i.p.) injection of 2 X 10^6 LM-IL-2K^b/EO771 cells alone. The mice then received two subsequent immunizations at weekly intervals with 2 X 10^6 LM-IL-2K^b/EO771 cells i.p. and an equivalent number of LM-IL-2K^b/EO771 cells injected into the same breast as first injected, without additional EO771 cells. As a control, naive C57BL/6J mice were injected into the breast with EO771 cells alone, followed by the subsequent injections of growth media. As

additional controls, naive C57BL/6J mice were injected according to the same protocol with a mixture of EO771 cells and LM-IL-2K^b cells transfected with DNA from B16 melanoma cells (LM-IL-2K^b/B16), with EO771 cells and unmodified LM cells, or with EO771 cells and non tumor-DNA transfected LM-IL-2K^b cells. The results (Figure 2) indicate that the first appearance of tumor was significantly delayed ($p < .004$) in the group of mice injected with the mixture of EO771 cells and LM-IL-2K^b/EO771 cells, relative to that of mice in any of the other groups. Three mice in the group injected with EO771 cells and LM-IL-2K^b/EO771 cells failed to develop tumors and appeared to have rejected the breast cancer cells.

The development of resistance to EO771 cells in mice immunized with LM-IL-2K^b/EO771 cells was emphasized by the finding that the immunized mice survived significantly ($P < .01$) longer than mice in any of the various control groups, including mice injected with EO771 cells and LM-IL-2K^b cells transfected with DNA from B16 melanoma cells. Mice immunized with LM-IL-2K^b cells transfected with DNA from B16 cells failed to resist the growth of the breast cancer cells (Figure 3). In some instances, mice injected with EO771 cells and LM-IL-2K^b/EO771 cells

survived indefinitely, more than 110 days. The injections of LM-IL-2K^b/EO771 cells were without apparent harm. Tumors failed to form in mice injected with LM-IL-2K^b/EO771 cells alone. Since LM cells express foreign histocompatibility determinants in C57BL/6J mice, it is likely that, like other foreign tissue grafts, the cells were rejected.

To determine if the injections of LM-IL-2K^b/EO771 cells resulted in long-term, generalized immunity toward the breast cancer cells, surviving mice in the group immunized with EO771 cells and LM-IL-2K^b/EO771 cells received a second injection of EO771 cells 110 days after the first immunization. The presence of generalized, long-term immunity to the breast cancer cells was indicated by the finding mice injected a second time with EO771 cells survived significantly ($P < .02$) longer than naive mice injected with an equivalent number of EO771 cells alone ((33 ± 6 days and 20 ± 6 days respectively) (Figure 4).

CD8$^+$ Cells Mediate Immunity to Breast Cancer in Mice Immunized with Fibroblasts Transfected with DNA from Breast Cancer Cells.

T cell-depletion was used to determine the subset of T cells that mediated resistance to tumor growth in mice immunized with the DNA-transfected cells. In the experiment, T cell-depletion was accomplished by injecting C57BL/6J mice i.p. with $CD8^+$ or $CD4^+$ monoclonal antibodies, as described in the Materials and Methods section. Two days later, the mice received a second injection of the antibodies followed by an injection into the fat pad of the breast with a mixture of 5 X 10^3 E0771 cells and 2 X 10^6 LM-IL-$2K^b$/EO771 cells. The mice received two subsequent injections of equivalent numbers of LM-IL-$2K^b$/EO771 cells and additional injections of the monoclonal antibodies, as described. As indicated (Figure 5), the first appearance of tumor and survival of immunized mice depleted of $CD8^+$ cells was not significantly different than the first appearance of tumor and survival of mice injected with E0771 cells alone. Depletion of $CD4^+$ cells had no apparent effect on resistance to tumor growth. The first appearance of tumor and survival of $CD4^+$ T cell-depleted mice injected with EO771 cells and LM-IL-$2K^b$/EO771 cells was not significantly different than the first appearance of tumor and survival of mice injected with EO771 cells and LM-IL-$2K^b$/EO771 cells alone. Thus, depletion of $CD8^+$ T cells, but not $CD4^+$

cells, affected the animals' capacity to resist the growth of the breast cancer cells in mice immunized with the DNA-transfected cells.

CD8$^+$ T cell-depletion had analogous effects on the survival of mice injected with the breast cancer cells and the DNA-transfected fibroblasts. As indicated (Figure 6), the survival of CD8$^+$-depleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells was significantly ($P < .01$) less than the survival of non depleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells alone. It was not significantly different than the survival of non T cell-depleted mice injected with EO771 cells alone. In contrast, depletion of CD4$^+$ cells had no effect on survival. The survival of mice depleted of CD4$^+$ cells injected with EO771 cells and LM-IL-2K^b/EO771 cells was not significantly different than that of non T cell-depleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells alone (Figure 6).

Thus, depletion of CD8$^+$ but not CD4$^+$ cells affected both tumor growth and survival of the immunized mice with breast cancer.

Survival of C3H/HeJ Mice Injected with a Mixture of Cells from a Spontaneous Adenocarcinoma of the Breast (SB-1) and LM-IL-2K^b cells Transfected with DNA from SB-1 cells.

Specific partial immunity toward EO771 cells, a breast cancer cell line, was generated in C57BL/6J mice immunized with semi-allogeneic, IL-2-secreting mouse fibroblasts transfected with DNA from EO771 cells. The same protocol was followed to determine if an analogous response would be obtained in mice immunized with the modified fibroblasts transfected with DNA taken directly from a spontaneous breast adenocarcinoma arising in a C3H/HeJ mouse.

C3H/HeJ mice develop breast cancer spontaneously. A tumor that developed in the breast of a 12 month old mouse was excised and used as a source of DNA to develop the vaccine. Histologic sections indicated that it was an adenocarcinoma. In addition, naive C3H/HeJ mice had no apparent resistance to the growth of the breast cancer cells. One hundred percent of mice injected with 1 X 10^4 SB-1 cells into the fat pad of the breast died from progressive tumor growth in approximately 30 days.

The effect of immunization with LM-IL-2K^b cells transfected with DNA from the spontaneous breast neoplasm (SB-1 cells) on the growth of the breast cancer cells was determined by injecting naive C3H/HeJ mice into the fat pad of the breast with SB-1 cells and LM-IL-2K^b/SB-1 cells, and i.p. with LM-IL-2K^b/SB-1 cells alone. As previously, the mice received two subsequent injections i.p. and two subsequent injections into the same breast as first injected with the same number of LM-IL-2K^b/SB-1 cells. The results (Figure 7) indicated that the time to first appearance of a palpable tumor in the breasts of mice injected with the mixture of SB-1 cells and LM-IL-2K^b/SB-1 cells was significantly delayed ($p < .006$), relative to the first appearance of tumor in mice injected with SB-1 cells alone. Once the breast neoplasms first appeared, the rate of tumor growth (two dimensional measurements) in the treated and untreated groups was approximately the same.

Consistent with the delayed appearance of tumor in the treated group, mice injected with SB-1 cells and LM-IL-2K^b/SB-1 cells survived significantly ($P < .006$) longer than mice injected with SB-1 cells alone (Figure 8). No tumors formed at immunization sites injected with LM-IL-2K^b/SB-1 cells alone.

As controls, naive C3H/HeJ mice were injected according to the same protocol with SB-1 cells and non transfected LM-IL-2 cells, with SB-1 cells and non transfected LM-IL-2K^b cells, or with SB-1 cells and syngeneic LM-IL-2 cells transfected with DNA from SB-1 cells (LM-IL-2/SB-1). As indicated (Figures 7 and 8), with the exception of two mice in the group injected with SB-1 cells and LM-IL-2K^b/EO771 cells, the first appearance of tumor, rate of tumor growth and survival of mice in each group was approximately the same as that of mice injected with SB-1 cells alone. Thus, the greatest immunotherapeutic benefit was in the group of mice injected with the mixture of SB-1 cells and semi allogeneic LM-IL-2K^b cells transfected with genomic DNA from SB-1 cells.

As a means of determining if immunizations with LM-IL-2K^b cells transfected with DNA from EO771 cells conferred immunity to SB-1 cells, naive C3H/HeJ mice were injected with a mixture of SB-1 cells and LM-IL-2K^b/EO771 cells. As indicated (Figure 8), although mice injected with SB-1 cells and LM-IL-2K^b/EO771 cells survived longer than mice injected with SB-1 cells alone, they died in significantly ($P < .01$) shorter

intervals than mice injected with SB-1 cells and LM-IL-2K^b cells transfected with DNA from the same breast cancer.

Discussion

The extraordinarily high incidence of breast cancer in women, approximately one in eight will develop the disease at some point in her life, created an urgent need for new and innovative forms of therapy. Immunotherapeutic approaches, designed to stimulate immunity to autologous tumor, are under active investigation for a number of different types of cancers. The theoretical basis underlying this form of treatment is that neoplastic cells form unique TAAs that can be recognized by cytotoxic T lymphocytes, and that cellular immunity to TAAs can follow immunization with tumor vaccines. Malignant cells in the patient can become targets of immune-mediated attack. Like other neoplasms, breast cancer cells form TAAs, several of which have been identified (5-8). However, antigens associated with the proliferating malignant cells are insufficiently immunogenic to generate an effective immune response. Proliferating breast cancer cells fail to elicit anti tumor immune responses that can control tumor cell-growth.

Here, we transferred high molecular weight DNA from breast cancer cells into a mouse fibroblast cell line to develop a breast cancer

vaccine that was effective in the treatment of breast cancer in mice. This approach was based on prior studies that indicated that the introduction of high molecular weight genomic DNA from one cell-type altered the genotype and the phenotypic characteristics of the cells that took up the exogenous DNA. This was the case for transfer of single genes specifying enzymes or membrane receptors (44-46). The gene-products were expressed by subpopulations of the transfected cells. In an analogous manner, the antigenic properties of weakly immunogenic tumor antigens associated with the breast cancer cells were enhanced by transfer of breast cancer DNA into a highly immunogenic cell line.

A fibroblast cell line was chosen as the platform for expression of the breast cancer-associated antigens, for several important reasons. The cells, maintained in vitro, were readily transfected, using conventional laboratory procedures. And, since the exogenous DNA is replicated as the cells divide, the number of transfected cells was expanded as was required for multiple immunizations of the tumor-bearing mice. In addition, like dendritic cells, fibroblasts can act as efficient antigen-presenting cells (53, 54). They express B7.1, a co stimulatory molecule required for T cell activation constiutively (55).

Class I cellular anti tumor immune responses were generated in tumor-bearing mice immunized with fibroblasts transfected with tumor-DNA (56, 57).

In this study, DNA was isolated from an adenocarcinoma of the breast that arose spontaneously in a C3H/HeJ mouse ($H\text{-}2^k$). DNA from the breast cancer cells was used to transfect LM cells, a mouse fibroblast cell line of C3H/He mouse origin. Before they were transfected with the tumor-DNA, to increase their non specific immunogenic properties, and to ensure rejection, the fibroblasts were modified to express foreign (allogeneic) $H\text{-}2K^b$-determinants, and to secrete IL-2. CTL-mediated anti tumor immune responses were generated in mice immunized with the transfected cells. The first appearance of tumor was delayed and the mice survived significantly longer than mice in various control groups, including mice injected with the breast cancer cells and transfected fibroblasts that formed syngeneic MHC-determinants alone.

An analogous study was carried out using IL-2-secreting LM fibroblasts modified to express $H\text{-}2K^b$-determinants that were transfected with DNA from EO771 cells, a breast cancer cell line

originating in C57BL/6J mice. H-2K^b-determinants are syngeneic class I MHC-determinants in C57BL/6J mice, providing a restriction-element for direct antigen presentation to CTLs of the host (53). Like the survival of C3H/HeJ mice with breast cancer treated by immunization with fibroblasts transfected with breast cancer DNA, C57BL/6J mice injected with EO771 cells and LM-IL-2K^b cells transfected with DNA from EO771 cells survived significantly longer than mice in various control groups, including mice injected with EO771 cells and modified fibroblasts transfected with DNA from a mouse melanoma cell line, an unrelated tumor. Some of the mice immunized with the DNA-transfected fibroblasts appeared to have rejected the breast cancer cells and survived indefinitely. Immunity failed to develop in mice depleted of CD8$^+$ cells indicating the essential role of this subset of T cells in mediating tumor rejection.

We conclude that modified fibroblasts that were transfected with the breast cancer DNA formed an array of undefined breast cancer associated antigens. No attempt was made to identify TAAs expressed by the transfected cells. The identification of tumor antigens is technically challenging and may not be required in the treatment of breast cancer

patients. In addition, the use of one or several defined antigens for immunotherapy may not eliminate the entire malignant cell population as some tumor cells in the population may fail to express the antigen chosen for immunization.

Transfection of tumor DNA into a highly immunogenic cell line has other important advantages. The amount of tumor DNA required to prepare the vaccine can be small, since the transferred DNA is replicated as the cells divide. A tumor cell line does not have to be established, if the patient's own tumor is to be used for immunotherapy. Tumor DNA can be readily obtained from primary neoplasms. Furthermore, the cells used as recipients of the tumor-DNA can be modified in advance for special properties, such as identity with the patient for shared class I determinants, or to secrete one or more cytokines, to further augment their immunogenic properties.

Surprisingly, the number of transfected cells that expressed the products of genes specifying TAAs was sufficient to induce the anti tumor immune response. Our observation that anti tumor immune responses followed immunizations with the transfected cells may be an

indication that multiple, and possibly large numbers of immunologically distinct TAAs, the products of multiple altered genes, were present within the population of breast cancer cells.

The results reported here raise the possibility that a human fibroblast cell line that shares identity at one or more MHC class I alleles with the cancer patient may be readily modified to provide immunologic specificity for TAAs expressed by the patient's neoplasm. The data suggest that an optimum response can be obtained if the cellular immunogen is prepared using DNA from the patient's own tumor. Transfection of the cell line with DNA from the neoplastic cells may provide a practical alternative to the modification of autologous malignant cells for the purposes of generating an immunogen that is useful in the overall management of the patient's disease.

References

1. Boon T, Cerottini J-C, Van den Eynde B, van der Bruggen P, Van Pel A. Tumor antigens recognized by T lymphocytes. Ann. Rev. Immunol. 1994;12:337-52.

2. Boon T. Tumor antigens recognized by cytolytic T lymphocytes: present perspectives for specific immunotherapy. Int. J. Cancer 1993;54:177-85.

3. Boon T. Toward a genetic analysis of tumor rejection antigens. Advances Cancer Res. 1992;58:177-91.

4. Storkus WJ, Zeh HJ III, Maeurer MJ, Salter RD, Lotze MT. Identification of human melanoma peptides recognized by class I restricted tumors infiltrating T lymphocytes. J. Immunol. 1993;151:3719-24.

5. Disis ML, Calenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, et al. Existent T cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res. 1994;54:16-20.

6. Brasseur F, Marchand M, Vanwijk R, Herin M, Lethe B, Chomez P. and Boon T. Human gene MAGE-1 which codes for a tumor rejection antigen is expressed in some breast tumors. Int. J. Cancer. 1992;52:839-41.

7. Fujie T, Mori M, Ueo H, Sugimachi K, Akiyoshi, T. Expression of MAGE and BAGE genes in Japanese breast cancers. Ann Oncol. 1997;8:369-72.

8. Horgan PG, Byrne J, O'Donoghue, J, Mooney E, Grimes H, Given HF. Mucin-like carcinoma associated antigen (MCA) at presentation with breast cancer. Ir J Med Sci. 1997;166:215-6.

9. Domenenech N, Henderson RA, Finn OJ. Identification of an HLA-A11-restricted epitope from the tandem repeat domain of the epithelial tumor antigen mucin. J. Immunol. 1995;155:4766-74.

10. Jerome KR, Domenech N, Finn OJ. Tumor-specific cytotoxic T lymphocyte clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin cDNA. J. Immunol. 1992;151:1644-59.

11. Wooster R, Cleton-Jansen A, Collins N, Mangion J, Cornelius R, Cooper C, et al. Instability of short tandem repeats (microsatellites) in human cancers. Nature Genet. 1994; 6:152-5.

12. Merlo A, Mabry M, Gabrielson E, Vollmer R, Baylin SB, Sidransky D. Frequent microsatellite instability in primary small lung cancer. Cancer Res. 1994; 54:2098-104.

13. Orth K, Hung J, Gazdar A, Bowcock A, Mathis JM, Sambrook J. Genetic instability in human ovarian cancer cell lines. Proc. Natl. Acad. Sci. (USA) 1994; 91:9495-99.

14. Parsons R, Li G-M, Longley MJ, Fang W-H, Papadopoulos N, Jen J, et al. Hypermutability and mismatch repair deficiency in RER+ tumor cells. Cell. 1993;75:1227-34.

15. Shibata D, Peinado MA, Ivonov Y, Malkhosyan S, Perucho M. Genomic instability in repeated sequences is an early somatic event in colorectal

tumorigeneis that persists after transformation. Nature Genetics. 1994; 6:273-76.

16. Specht JM, Wang G, Do MT, Lam JS, Royal RE, Reeves, ME, et al. Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases. J Exp Med. 1997:186:1213-21.

17. Kim CJ, Prevette T, Cormier J, Overwijk W, Roden M, Restifo NP. Dendritic cells infected with poxviruses encoding MART-1/Melan A sensitize T lymphocytes in vitro. J Immunother. 1997;20:276-86.

18. Rosenberg SA, Cancer vaccines based on the identification of genes encoding cancer regression antigens. Immunol Today 1997;18:175-182.

19. Tuting T, Storkus WJ, Lotze MT. Gene-based strategies for the immunotherapy of cancer. J Mol Med., 1997;75:478-91.

20 Shurin MR, Esche C, Peron JM, Lotze MT. Antitumor activities of IL-12 and mechanisms of action. Chem Immunol., 1997;68:153-74.

21. Nabel GJ, Gordon D, Bishop DK, Nickoloff BJ, Yang Z-Y, Aruga A, et al. Immune response in human melanoma after transfer of an allogeneic class I major histocompatibility complex gene with DNA-liposome complexes. Proc. Natl. Acad. Sci. (USA), 1996;93:15388-93.

22. Kawakami Y and Rosenberg SA. Immunobiology of human melanoma antigens MART-1 and gp100 and their use for immuno-gene therapy. Int Rev Immunol., 1997;14:173-92.

23. Cavallo F, Pierro FD, Giovarelli M, Gulino A., Vacca A, Stoppacciaro A, et al. Protective and curative potential of vaccination with interleukin-2-gene-transfected cells from a spontaneous mouse mammary adenocarcinoma. Cancer Res., 1993;53:5067-73.

24. Russell SJ, Eccles SA, Flemming CL, Johnson CA, Collins MKL. Decreased tumorigenicity of a transplantable rat sarcoma following transfer and expression of an IL-2 cDNA. Int. J. Cancer, 1991;47:244-49.

25. Chakravarty PK, Fuji H, Abu-hadid MM, Hsu S-C, Sood AK. Tumorigenicity of interleukin-2 (IL-2)-cDNA-transfected L1210 lymphoma and its in vivo variants is modulated by changes in IL-2 expression: potential therapeutic implications. Cancer Immunol. Immunother. 1992;35:347-51.

26. Kim TS, Russell SJ, Collins MKL, Cohen EP. Immunization with interleukin-2-secreting allogeneic mouse fibroblasts expressing melanoma-associated antigens prolongs the survival of tumor-bearing mice with melanoma. Int. J. Cancer, 1993;55:865-72.

27. Kim TS, Cohen EP. Immunization of mice with allogeneic fibroblasts genetically modified for interleukin-2-secretion and expression of melanoma-associated antigens stimulates predetermined classes of anti-melanoma effector cells. J. Immunotherapy, 1994;16:24-35.

28. Golumbek PT, Lazenby AJ, Levitsky HI, Jaffee LM, Karasuyama H, Baker M, et al. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. Science, 1991;254:713-16.

29. Mullen CA, Coale MM, Levy AT, Stetler-Stevenson WG, Liotta LA, Brandt S, et al. Fibrosarcoma cells transduced with the IL-6 gene exhibit reduced tumorigenicity, increased immunogenicity, and decreased metastatic potential. Cancer Res., 1992;52:6020-6026.

30. Aoki T, Tashiro K, Miyatake S-I, Kinashi T, Nakano T, Oda Y, Kikuchi H, Honjo T. Expression of murine interleukin 7 in a murine glioma cell-line results in reduced tumorigenicity *in vivo.* Proc. Natl. Acad. Sci. (USA), 1992;89:3850-54.

31. Tahara H, Zeh HJ, Storkus WJ, Pappo I, Watkins SC, Gubler U, et al. Fibroblasts genetically engineered to secrete interleukin 12 can suppress tumor growth and induce antitumor immunity to a murine melanoma in vivo. Cancer Res., 1994;54:182-87.

32. Asher AL, Mule JJ, Kasid A. Murine tumor cells transduced with the gene for tumor necrosis factor-α: evidence for paracrine immune effects of tumor necrosis factor against tumors. J. Immunol. 1991;146:3227-33.

33. Blankenstein T, Qin Z, Uberla K, Muller W, Rosen H, Volk, H.-D, et al. Tumor suppression after tumor cell-targeted tumor necrosis factor α gene transfer. J. Exp. Med. 1991;173:1047-55.

34. Ferrantini M, Proietti E, Santodonato L, Gabriele L, Peretti M, Plavec I, et al. α1-interferon gene transfer into metastatic Friend leukemia cells abrogated tumorigenicity in immunocompetent mice: antitumor by means of interferon-producing cells. Cancer Res. 1993;53:1107-12.

35. Gansbacher B, Bannerji R, Daniels B, Zier K, Cronin K, Gilboa E. Retroviral vector-mediated γ-interferon gene transfer into tumor cells generates potent and long lasting antitumor immunity. Cancer Res. 1990 50:7820-24.

36. Columbo MP, Ferrari G, Stoppacciaro A, Parenza M, Rodolfo M, Mavilio F, et al. Granulocyte colony-stimulating factor gene transfer suppresses tumorigenicity of a murine adenocarcinoma *in vivo.* J. Exp. Med. 1991;173: 889-95.

37. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, et al. Vaccination with irradiated tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc. Natl. Acad. Sci. (USA). 1991;90:3539-43.

38. Bottazzi B, Walter S, Govoni D, Colotta F. Mantovani A. Monocyte chemotactic cytokine gene transfer modulates macrophage infiltration, growth, and susceptibility to IL-2 therapy of a murine melanoma. J. Immunol. 1992;148:1280-85.

39. Itaya T, Yamagima S, Okada F, Oikawa T, Kuzumaki N, Takeichi N, Hosokawa M, Kobyashi H. Xenogenization of mouse lung carcinoma (3LL) by transfection with an allogeneic class I major histocompatibility complex gene (H-2L^{d}). Cancer Res. 1987;47:3136-42.

40. Mandelboim O, Vadai E, Feldman M, Eisenbach L. Expression of two H-2K genes, syngeneic and allogeneic, as a strategy for potentiating immune recognition of tumor cells. Gene Ther. 1995; 2:757-80.

41. Hui KM, Sim TF, Foo TT, Oei A-A. Tumor rejection mediated by transfection with allogeneic class I histocompatibility gene. J. Immunol. 1989;143:3835-41.

42. Ostrand-Rosenberg S, Thakur A, Clements V. Rejection of mouse sarcoma cells after transfection of MHC class II genes. J. Immunol. 1990;144:4068-72.

43. Dubey C, Croft M, Swain SL. Co-stimulatory requirements fo naive CD4+ T-cells: ICAM-1 or B-7-1 can co stimulate naive CD4 T cell activation but both are required for optimum response. J Immunol. 1995;155:45-51.

44. Wigler M, Pellicer A, Silverstein S, Axel R, Urlaub G, Chasin L. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc Natl Acad Sci (USA). 1979;76:1373-76.

45. Wigler M, Pellicer A, Silverstein S, Axel R. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell 1978;14:725-31.

46. Mendersohn, C., Johnson, B., Lionetti, K.A., Nobis, P., Wimmer, E. and Racaniello, V.R. Transformation of a human poliovirus receptor gene into mouse cells. Proc Natl Acad Sci (USA). 1986;83:7845-49.

47. Gillis S, Ferm MM, Ou W, Smith KA. T-cell growth factor: parameters of production and quantitative microassay for activity. J. Immunol. 1978;120:2027-34.

48. Allen H, Wraith D, Pala P, Askonas B, Flavell R A. Domain interactions of H-2 class I antigens alter cytotoxic T-cell recognition sites. Nature 1984;309:279-82.

49. Vile R, Hart I. In vitro and in vivo targeting of gene expression to melanoma cells. Cancer Res. 1993;53:962-67.

50. Wilde DB, Marrack P, Kappler J, Dialnas DP, Fitch FW. Evidence implicating L3T4 in class II MHC antigen reactivity; monoclonal antibody GK1.5 (anti-L3T4a) blocks class II MHC antigen-specific proliferation,

release of lymphokines, and binding by cloned murine helper T lymphocyte lines. J Immunol 1983;131:2178-83.

51. Sarmiento M, Glasebrook AL, Fitch FW. IgG or IgM monoclonal antibodies bearing Lyt2 antigen block T cell-mediated cytolysis in the absence of complement. J Immunol. 1980;125:2665-2670.

52. Sugden B, Marsh K, Yates J. A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. Mol. Cell Biol. 1985;5:410-16.

53. Kundig TM, Bachmann MF, DiPaolo C, Simard JJL, Battegay M, Lother H, et al. Fibroblasts as efficient antigen-presenting cells in lymphoid organs. Science. 1995;268:1343-45.

54. Schoenberger SP, Jonges LE, Mooijaart RJD, Hartgers F, Toes REM, Kast WM, Melief CJM, Offringa R. Efficient direct priming of tumor-specific cytotoxic T lymphocytes in vivo by an engineered APC. Cancer Res. 1998;3094-3100.

55. Kim TS, Xu WS, de Zoeten E, Cohen E P. Immunization with interleukin-2/interferon-g double cytokine-secreting allogeneic fibroblasts prolongs the survival of mice with melanoma. Melanoma Research. 1995;5:217-27.

56. Kim TS, Cohen EP. MHC antigen expression by melanomas recovered from mice treated with allogeneic mouse fibroblasts genetically modified for interleukin-2-secretion and the expression of melanoma associated antigens. Cancer Immunol. Immunother. 1994;38:185-91.

57. de Zoeten EF, Carr-Brendel VB, Cohen, E.P. Resistance to melanoma in mice immunized with semi-allogeneic fibroblasts transfected with DNA from mouse melanoma cells J. Immunol. 1998;160:2915-22.

FIGURE LEGENDS

Figure 1. The expression of H-2K^b-determinants by LM-IL-2 cells transduced with pBR327H-2K^b.

Legend: 1x10^4 LM-IL-2 cells transduced with the plasmid pBR327H-2K^b (LM-IL-2K^b cells) were incubated for 1 hr. at 4° with FITC-conjugated anti-H-2K^b, anti-H-2K^k, or anti-H-2K^d mAbs, or mouse IgG$_{2a}$ (isotype control) as described in the Materials and Methods section. The cells were then analyzed for fluorescent staining by quantitative flow cytofluorography.

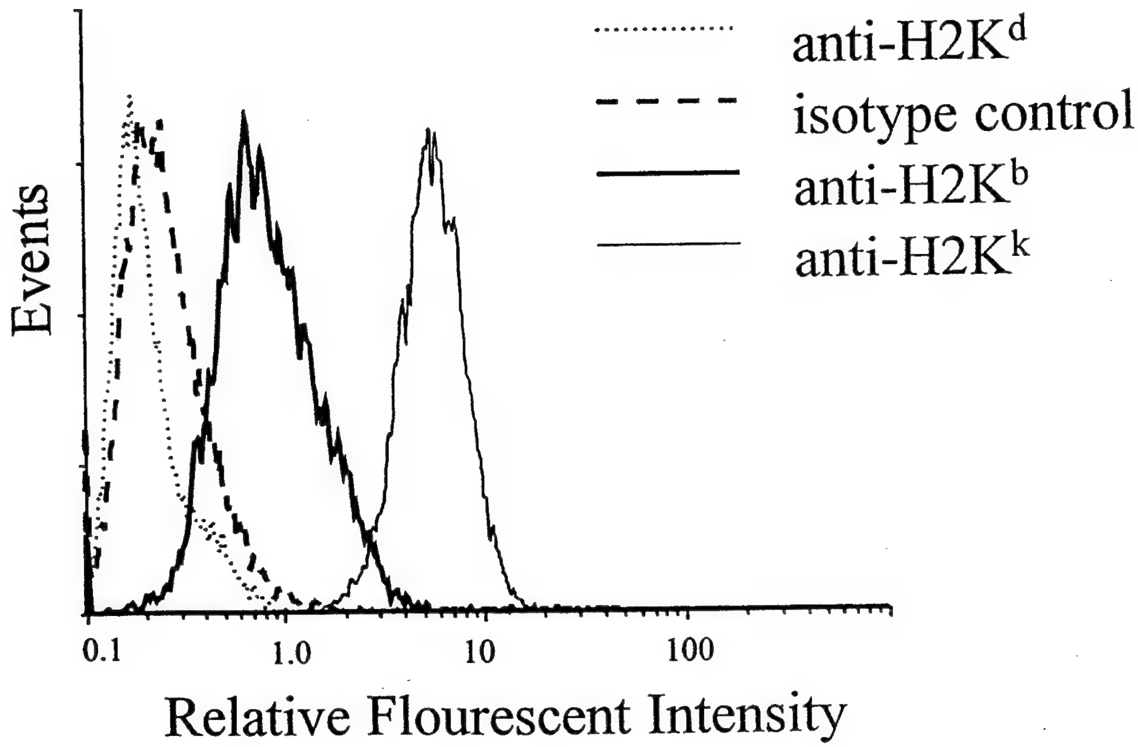

anti-H2K^d
isotype control
anti-H2K^b
anti-H2K^k
Events
0.1
1.0
10
100
Relative Flourescent Intensity

Figure 1

Figure 2. Tumor growth in C57BL/6J mice injected with a mixture of EO771 breast cancer cells and LM-IL-2K^b/EO771 cells.

Legend: C57BL/6J mice (7 per group) were injected into the fat pad of the breast with a mixture of 5 X 10^3 EO771 breast carcinoma cells and 2 X 10^6 LM-IL-2K^b/EO771 cells in a total volume of 200 ul. At the same time the mice also received an injection i.p. of 2 X 10^6 LM-IL-2K^b/EO771 cells in 200 ul alone, followed by two subsequent injections at weekly intervals of 2 X 10^6 LM-IL-2K^b/EO771 cells i.p. and 2 X 10^6 LM-IL-2K^b/EO771 cells into the fat pad of the same breast as first injected. As controls, other naive C57BL/6J mice were injected according to the same protocol with equivalent numbers of EO771 cells and unmodified LM cells, with EO771 cells and LM-IL-2K^b cells, with EO771 cells and LM-IL-2K^b/B16 cells or with EO771 cells into the breast alone, followed by subsequent injections of growth media.

Mean tumor volume was derived from two dimensional measurements obtained with a dial caliper. The volume is equal to 0.4ab^2, where a = length, and b = width.

P < .006 for difference in survival of mice injected with E0771 cells and LM cells, and mice injected with E0771 cells and LM-IL-2K^b/EO771 cells.

P < .01 for difference in survival of mice injected with EO771 cells and LM-IL-2K^b cells, and mice injected with E0771 cells and LM-IL-2K^b/EO771 cells.

P < .03 for difference in survival of mice injected with EO771 cells and LM-IL-2K^b/B16 cells, and mice injected with E0771 cells and LM-IL-2K^b/EO771 cells.

Tumor volume was derived from two dimensional measurements obtained with a dial caliper. The volume is equal to $0.4ab^2$, where a = length, and b = width.

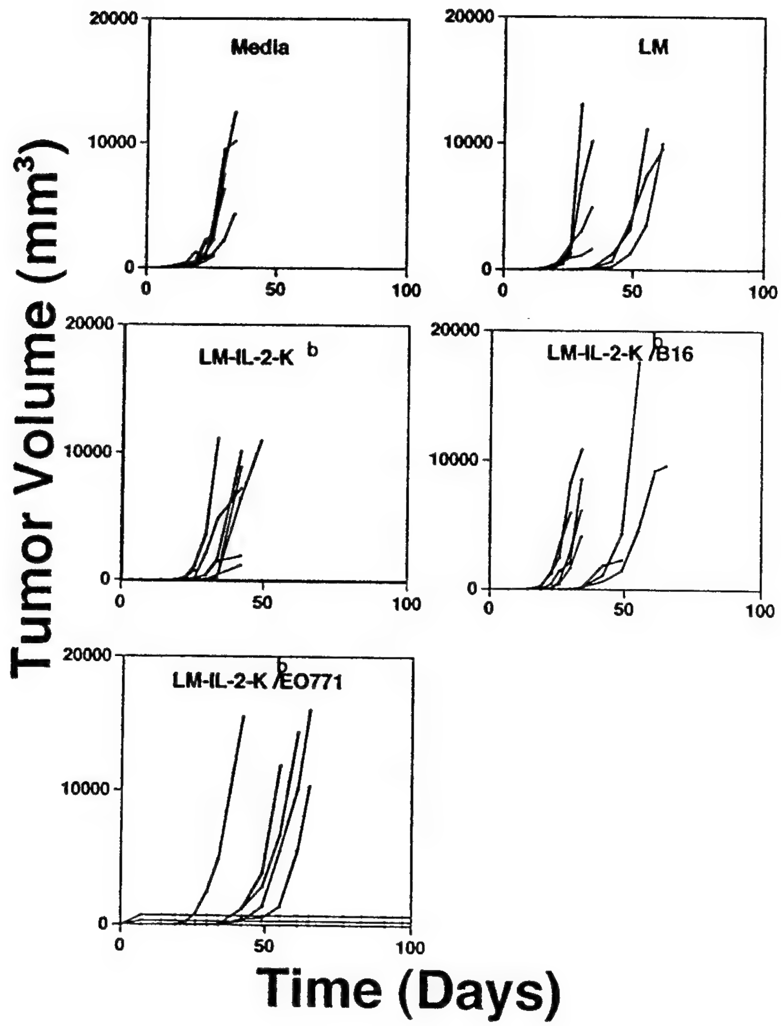

Tumor Volume (mm3)
Media
LM
LM-IL-2-K b
LM-IL-2-K b/B16
LM-IL-2-K b/EO771
20000
10000
0
50
100
Time (Days)

Figure 2

Figure 3. Survival of C57BL/6J mice injected with a mixture of EO771 breast carcinoma cells and LM-IL-2K^b/EO771 cells.

Legend: C57BL/6J mice (7 per group) were injected into the fat pad of the breast with a mixture of 5 X 10^3 EO771 breast carcinoma cells and 2 X 10^6 LM-IL-2K^b/EO771 cells in a total volume of 200 ul. At the same time the mice also received an injection i.p. of 2 X 10^6 LM-IL-2K^b/EO771 cells in 200 ul alone, followed by two subsequent injections at weekly intervals of 2 X 10^6 LM-IL-2K^b/EO771 cells i.p. and 2 X 10^6 LM-IL-2K^b/EO771 cells into the fat pad of the same breast as first injected. As controls, other naive C57BL/6J mice were injected according to the same protocol with equivalent numbers of EO771 cells and LM cells, with EO771 cells and LM-IL-2K^b cells, with EO771 cells and LM-IL-2K^b/B16 cells or with EO771 cells into the breast alone, without subsequent injections.

Mean survival times: Mice injected with viable EO771 cells alone 34.5 ± 5.8 days; mice injected with viable EO771 cells and LM cells, 41 ± 14 days; mice injected with viable EO771 cells and LM-IL-2K^b cells, 44 ± 9

days; mice injected with viable EO771 cells and LM-IL-2K^b/B16 cells, 46 ± 11 days; three mice injected with viable EO771 cells and LM-IL-2K^b/EO771 cells, > 110 days; MST for remaining mice dying from progressive tumor growth = 54 ± 9.

---- □ ---- injected with EO771 cells alone

---- ○ ---- injected with EO771 cells and LM cells

---- ● ---- injected with EO771 cells and LM-IL-2K^b cells

---- ▲ ---- injected with EO771 cells and LM-IL-2K^b/B16 cells

---- ■ ---- injected with EO771 cells and LM-IL-2K^b/EO771 cells.

P < .01 for difference in survival of mice injected EO771 cells, and mice injected with E0771 cells and LM-IL-2K^b/EO771 cells.

P < .01 for difference in survival of mice injected with E0771 cells and LM cells, and mice injected with E0771 cells and LM-IL-2K^b/EO771 cells.

P < .01 for difference in survival of mice injected with EO771 cells and LM-IL-2K^b cells, and mice injected with E0771 cells and LM-IL-2K^b/EO771 cells.

$P < .01$ for difference in survival of mice injected with EO771 cells and LM-IL-2K^b/B16 cells, and mice injected with E0771 cells and LM-IL-2K^b/EO771 cells.

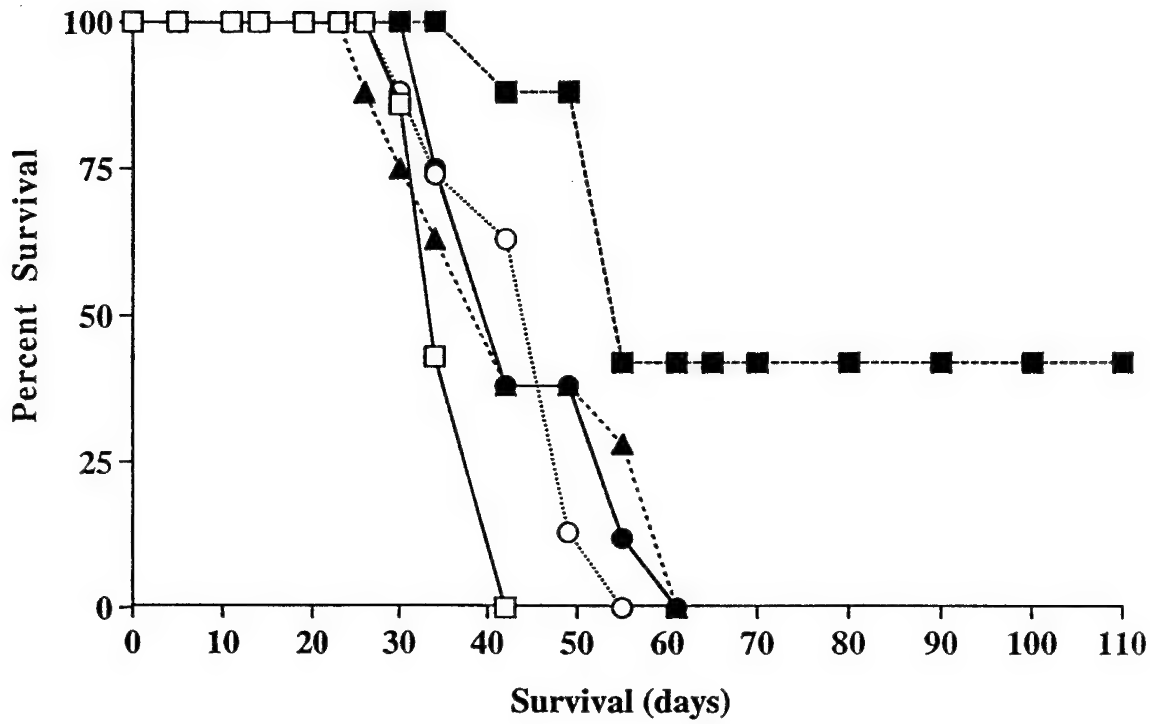

Percent Survival
100
75
50
25
0
0
10
20
30
40
50
60
70
80
90
100
110
Survival (days)

Figure 3

Figure 4. Survival of C57BL/6J mice surviving a prior injection of EO771 cells and LM-IL-2K^b/EO771 cells injected with EO771 cells alone.

Legend: Three C57BL/6J mice surviving 110 days after the prior injection of EO771 cells and LM-IL-2K^b/EO771 cells were injected into the fat pad of the breast a second time with 5 X 10^3 EO771 cells alone. As a control, five naive C57BL/6J mice were injected into the fat pad of the breast with an equivalent number of EO771 cells. $P < .02$ for the difference in survival of mice in the two groups.

---- ● ---- Surviving mice injected with EO771 cells

---- □ ---- Naive mice injected with EO771 cells

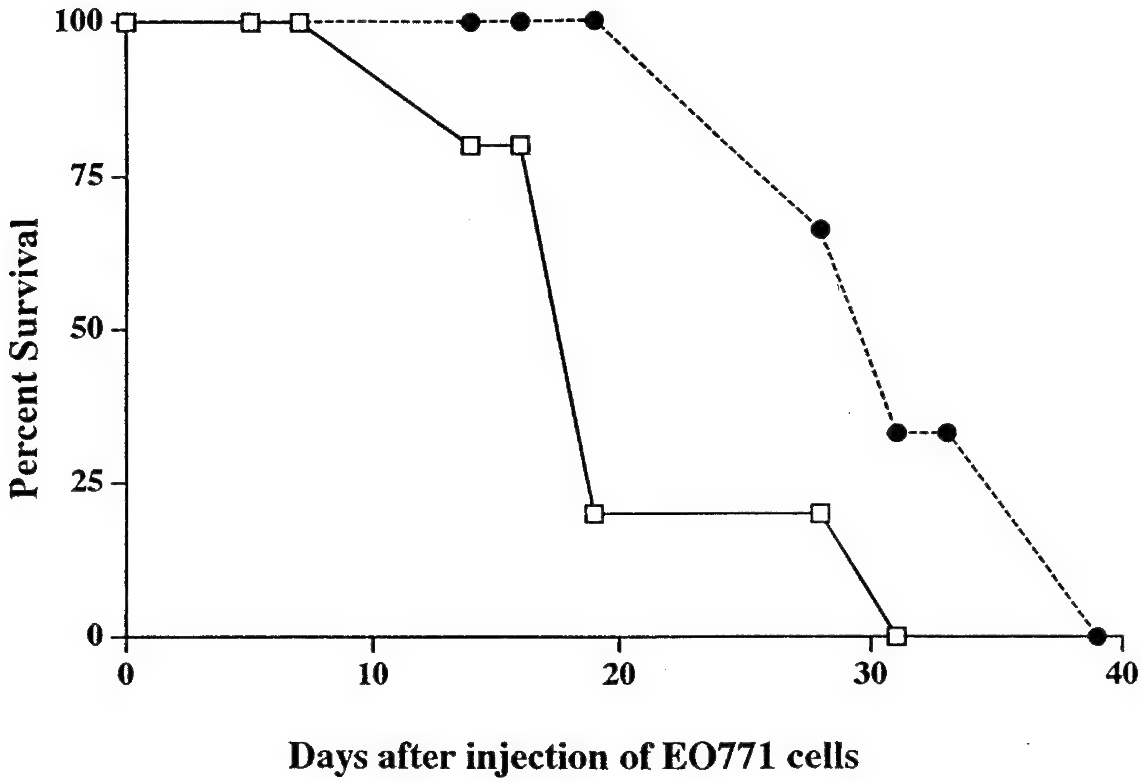

100
75
50
25
0
Percent Survival
0
10
20
30
40
Days after injection of EO771 cells

Figure 4

Figure 5. Tumor growth in C57BL/6J mice depleted of $CD8^+$ T lymphocytes injected with a mixture of EO771 breast cancer cells and LM-IL-2K^b/EO771 cells.

Legend: C57BL/6J mice (7 per group) were injected i.p. with $CD8^+$ or $CD4^+$ monoclonal antibodies, as described in the Materials and Methods section. Two days later, the were injected into the fat pad of the breast with a mixture of 5 X 10^3 EO771 breast carcinoma cells and 2 X 10^6 LM-IL-2K^b/EO771 cells in a total volume of 200 ul. At the same time, the mice also received an injection i.p. of 2 X 10^6 LM-IL-2K^b/EO771 cells in 200 ul alone. The mice received additional injections of the relevant antibodies as described, and two subsequent injections at weekly intervals of 2 X 10^6 LM-IL-2K^b/EO771 cells i.p. and 2 X 10^6 LM-IL-2K^b/EO771 cells into the fat pad of the same breast as first injected. As controls, other C57BL/6J mice were injected according to the same protocol the monoclonal antibodies and equivalent numbers of EO771 cells cells into the breast alone, followed by subsequent injections of growth media. One group was injected with EO771 cells and LM-IL-2K^b/EO771 cells, but did not receive monoclonal antibodies.

Mean tumor volume was derived from two dimensional measurements obtained with a dial caliper. The volume is equal to $0.4ab^2$, where a = length, and b = width.

A = Injected with EO771 cells and LM-IL-2K^b/EO771 cells alone

B = Injected with $CD8^+$ antibodies, EO771 cells and LM-IL-2K^b/EO771 cells

C = Injected with $CD4^+$ antibodies, EO771 cells and LM-IL-2K^b/EO771 cells

D = Injected with EO771 cells alone

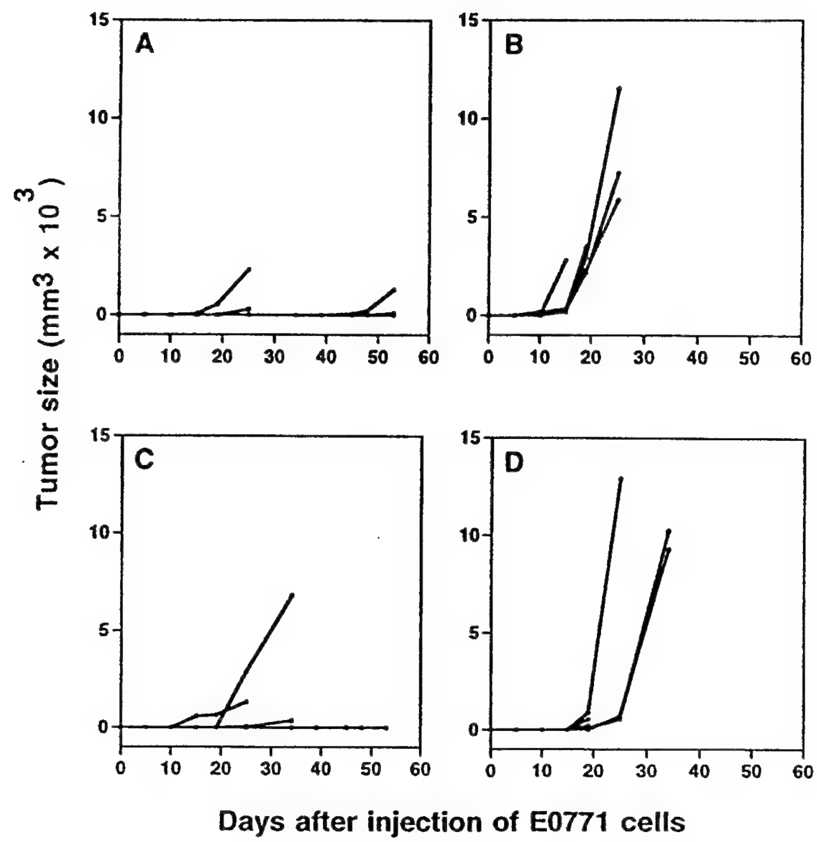

A
B
C
D
15
10
5
0
0
10
20
30
40
50
60
Tumor size (mm3 x 10^3)
Days after injection of E0771 cells

Figure 5

Figure 6. Survival of C57BL/6J mice depleted of T cells injected with a mixture of EO771 breast carcinoma cells and LM-IL-2K^b/EO771 cells.

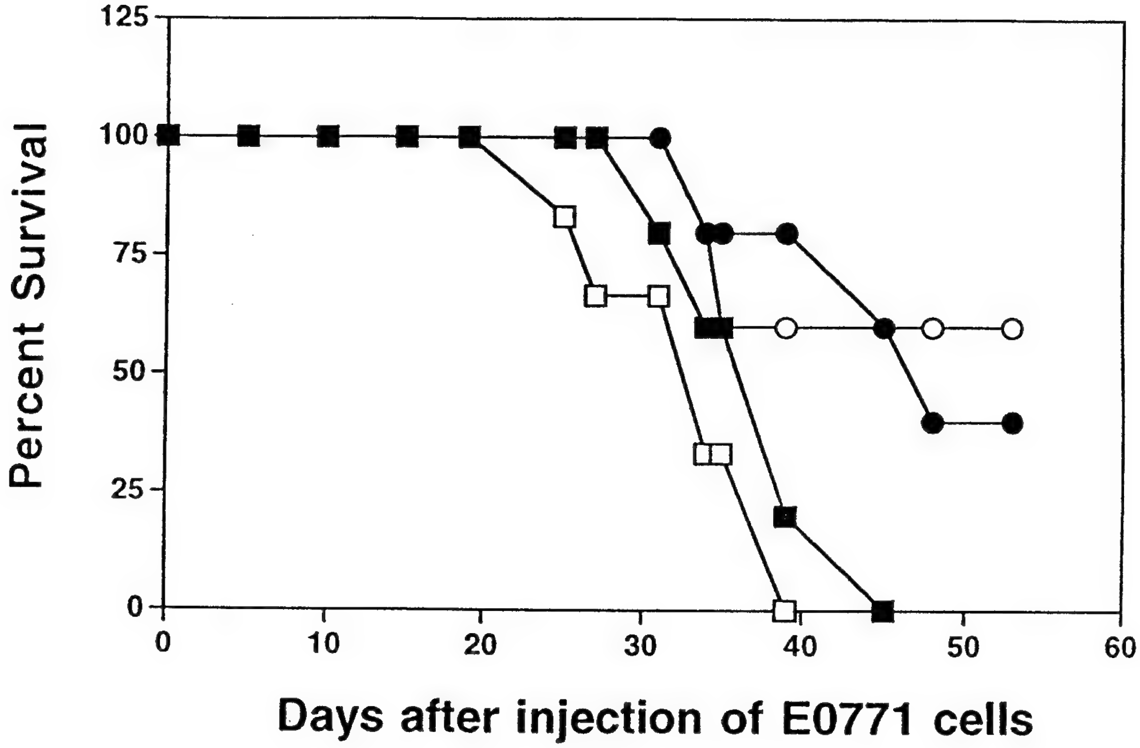
The same protocol as described in the Legend to Figure 5 was followed expect that survival of the antibody-treated mice was determined.

--- □ --- Injected with $CD8^+$ antibodies, EO771 cells and LM-IL-2K^b/EO771 cells

--- ● --- Injected with $CD4^+$ antibodies, EO771 cells and LM-IL-2K^b/EO771 cells

--- ■ --- Injected with EO771 cells alone

--- o --- Injected with EO771 cells and LM-IL-2K^b/EO771 cells alone



125
100
75
50
25
0
Percent Survival
0
10
20
30
40
50
60
Days after injection of E0771 cells

Figure 6

Figure 7. Tumor growth in C3H/HeJ mice injected with a mixture of cancer cells from a spontaneous breast neoplasm (SB-1), forming in a C3H/HeJ mouse, and LM-IL-2K^b transfected with DNA from SB-1 cells.

Legend: C3H/HeJ mice (5 per group) were injected into the fat pad of the breast with a mixture of 1 X 10^6 SB-1 cells and 2 X 10^6 LM-IL-2K^b/SB-1 cells. At the same time, the mice received an injection i.p. of 2 X10^6 LM-IL-2 K^b/SB-1 cells alone, followed by two subsequent injections. As controls, the mice were injected according to the same protocol with equivalent numbers of SB-1 cells alone, with SB-1 cells and LM-IL-2 cells, with SB-1 cells and LM-IL-2K^b cells, with SB-1 cells and LM-IL-2/SB-1 cells or with SB-1 cells and LM-IL-2K^b/EO771 cells. The mice were injected i.p. twice more, at weekly intervals, with the same number of modified cells as in the initial injections, but without additional SB-1 cells. Mean tumor volume was derived from two dimensional measurements obtained with a dial caliper.

Media = Mice injectd with SB1 tumor cells alone

LM-IL-2= Mice injected with a mixture of SB1 tumor cells an LM-IL-2 cells

LM-IL-2K^b= Mice injected with a mixture of SB1 tumor cells an LM-IL-2K^b cells

LM-IL-2/SB1= Mice injected with a mixture of SB1 tumor cells an LM-IL-2/SB1 cells

LM-IL-2K^b/SB1= Mice injected with a mixture of SB1 tumor cells an LM-IL-2K^b/SB1 cells

P < .006 for difference in survival of mice injected SB1 cells, and mice injected with SB1 cells and LM-IL-2K^b/SB1 cells.

P < .01 for difference in survival of mice injected with SB1 cells and LM-IL-2 cells, and mice injected with SB1 cells and LM-IL-2K^b/ SB1 cells.

$P < .005$ for difference in survival of mice injected with SB1 cells and LM-IL-2/SB1 cells, and mice injected with SB1 cells and LM-IL-2K^b/ SB1 cells.

$P < .02$ for difference in survival of mice injected with SB1 cells and LM-IL-2K^b cells, and mice injected with SB1 cells and LM-IL-2K^b/ SB1 cells.

$P < .03$ for difference in survival of mice injected with SB1 cells and LM-IL-2K^b/E0771 cells, and mice injected with SB1cells and LM-IL-2K^b/ SB1 cells.

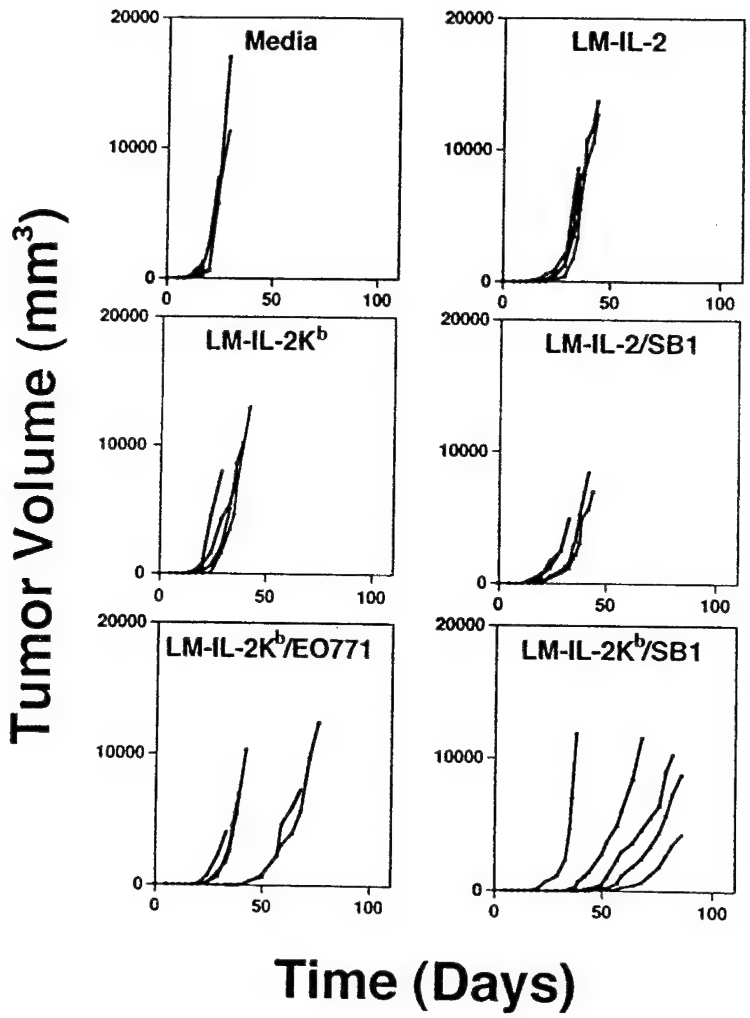

Tumor Volume (mm3)
Media
LM-IL-2
LM-IL-2Kb
LM-IL-2/SB1
LM-IL-2Kb/EO771
LM-IL-2Kb/SB1
20000
10000
0
0
50
100
Time (Days)

Figure 7

Figure 8. Survival of C3H/HeJ mice injected with a mixture of SB-1 breast carcinoma cells and LM-IL-2K^b/SB-1 cells.

Legend: C3H/HeJ mice (5 per group) were injected into the fat pad of the breast with a mixture of 5 X 10^3 SB-1 cells and 2 X 10^6 LM-IL-2K^b/SB-1 cells in a total volume of 200 ul. At the same time the mice received an injection i.p. of 2 X 10^6 LM-IL-2K^b/SB-1 cells in 200 ul alone, followed by two subsequent injections at weekly intervals of 2 X 10^6 LM-IL-2K^b/SB-1 cells i.p. and 2 X 10^6 LM-IL-2K^b/SB-1 cells into the fat pad of the same breast as first injected. As controls, other naive C57BL/6J mice were injected according to the same protocol with equivalent numbers of SB-1 cells and LM-IL-2 cells, with SB-1 cells and LM-IL-2K^b cells, with SB-1 cells and LM-IL-2/SB-1 cells, with SB-1 cells and LM-IL-2K^b/EO771 cells or with SB-1 cells into the breast alone, without subsequent injections.

Mean survival times: Mice injected with SB-1 cells alone 29 ± 7 days; with SB-1 cells and LM-IL-2 cells, 38 ± 8 days; with SB-1 cells and LM-IL-2K^b cells, 34 ± 7 days; with SB-1 cells and LM-IL-2/SB-1 cells, 36 ± 5 days; with SB-1 cells and LM-IL-2K^b/EO771 cells, 51 ± 18 days, with SB-1 cells and LM-IL-2K^b/SB-1 cells, 76 ± 26 days.

Survival of mice injected with SB-1 cells and LM-IL-$2K^b$/SB-1 cells, relative to survival of mice in each of the other groups, $p < .01$. $P < .02$ for difference in survival of mice injected with SB-1 cells alone and mice injected with SB-1 cells and LM-IL-$2K^b$/E0771 cells.

---- ■ ---- injected with SB-1 cells alone

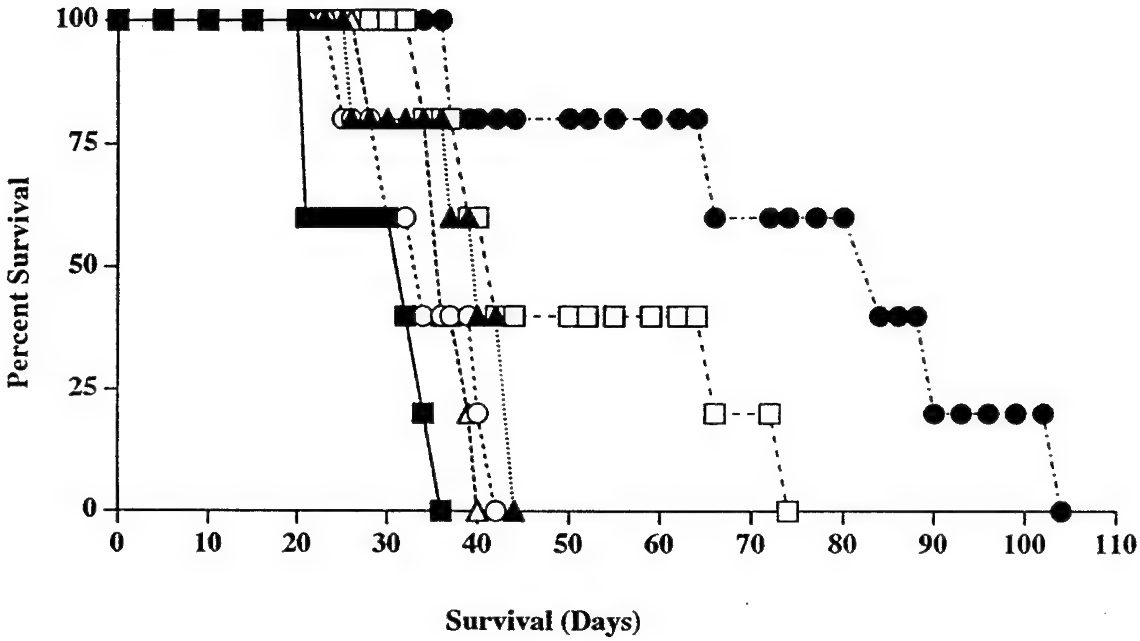
---- △ ---- injected with SB-1 cells and LM-IL-2 cells

---- ▲ ---- injected with SB-1 cells and LM-IL-$2K^b$ cells

---- ○ ---- injected with SB-1 cells and LM-IL-2/SB-1 cells

---- □ ---- injected with SB-1 cells and LM-IL-$2K^b$/EO771 cells

---- ● ---- injected with SB-1 cells and LM-IL-$2K^b$/SB-1 cells.



100
75
50
25
0
Percent Survival
0
10
20
30
40
50
60
70
80
90
100
110
Survival (Days)

Figure 8

Expression of B7.1 in a MUC1 expressing mouse mammary epithelial tumour cell line overcomes tolerance but does not induce autoimuunity in MUC1 transgenic mice

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Running Title :- B7.1 increases antigenicity of MUC1 without inducing auto-immunity

Summary

The MUC1 epithelial mucin, which is over-expressed and aberrantly glycosylated in breast and other carcinomas, is also expressed on the apical surface of most normal glandular epithelial cells. Since clinical trials evaluating the efficacy of MUC1 based vaccines have been initiated in breast cancer patients, it is important to address the question of whether an effective immune response to the cancer associated mucin can be generated without inducing auto-immunity. We have therefore looked at the effect of expressing B7.1 on the tumorigenicity of a MUC-1 expressing mammary epithelial cell line (410.4) in a transgenic mouse expressing MUC1 on its normal glandular epithelial tissues. Co-expression of B7.1 with MUC1 in 410.4 cells resulted in a dramatic inhibition of tumour growth which depended on the activity of CD4+ and CD8+ T cells. The epithelial tissues in the transgenic mice able to reject the B7.1, MUC1 expressing tumours showed no evidence of lymphocytic infiltration or degeneration and survived their normal life span. The results demonstrate that tolerance to the MUC1 antigen can be overcome without inducing auto-immunity. They also demonstrate that presentation of the MUC1 antigen, whether directly or by cross-priming, is markedly enhanced by expression of B7.1 by the MUC-1 expressing tumour cells.

Introduction

The human MUC1 gene codes for a type I membrane glycoprotein which is normally expressed on the apical surface of most glandular epithelial cells, but which is upregulated and underglycosylated in breast and other carcinomas (1). The extracellular domain of the MUC1 mucin is made up largely of exact tandem repeats of 20 amino acids (2), each repeat containing sites for O-linked glycosylation (3). The shorter carbohydrate side chains which are added in breast cancers result in the exposure of normally cryptic peptide epitopes, such as that recognised by the antibody SM3, and the creation of tumour-associated carbohydrate antigens (4). Both humoral and cellular responses to MUC1 have been detected in breast and other cancer patients (5-8), and MHC unrestricted cytotoxic T cell (CTL) responses have been reported (6,7) as well as classical MHC restricted responses (8). The repetitive structure and novel antigenic profile of the over-expressed cancer-associated mucin, together with the fact that T cell-mediated immune responses to this molecule can occur, has stimulated interest in the MUC1 glycoprotein as a target antigen for immunotherapy for carcinomas. Indeed several clinical studies using MUC1 based antigens have been initiated (8-12).

The CTLs isolated from breast and other cancer patients are anergic *in vivo*, and clearly none of the *in vivo* immune responses are effectively controlling tumour progression in cancer patients. One of the reasons put forward for the poor immune response to carcinomas is that they do not present antigen effectively to T cells because of the lack of expression of the co-stimulatory molecules expressed by professional antigen presenting cells. In this context it has been shown that introduction of a gene coding for co-stimulatory molecules such as B7.1 or B7.2 can dramatically enhance the immunogenicity of mouse tumour cell lines and thus reduce their tumorigenicity (13-18).

Since the MUC1 antigen is expressed by normal glandular epithelial cells, the question of whether auto-immunity would occur if the immune responses to the mucin were effective is a question which needs to be addressed. Since the dominant immunogenic domain of MUC1 is in the

tandem repeat sequence (2,19-22) which has a different sequence in the mouse homologue, muc1 (23), the use of the mouse for the necessary preclinical studiesis inappropriate, particularly for studies on auto-immunity. To address this and related questions, we have developed a transgenic mouse strain expressing the human MUC1 mucin on its glandular epithelial tissues with the same profile of expression as is seen in humans (24). Morover, the differences in glycosylation of MUC1 seen bewteen normal and malignant breast are also evident in the transgenic mouse: The SM3 epitope is minimally epressed on the mucin expressed by normal glandular tissues (24), but is expressed on both transplantable tumours expressing MUC1 (25) and on spontaneous tumours developing in transgenics cross fostered on an MMTV carrying mouse strain (26).

To ascertain whether an effective immune response to the MUC1 mucin can be obtained which inhibits tumour growth while not inducing autoimmunity, we have examined the effect of expressing the B7.1 co-stimulatory molecule on the tumorigenicity of a MUC1 expressing mouse mammary epithelial cell line in the MUC1 transgenic mice. Our results indicate that while co-expression of the B7.1 molecule decreases the tumorigenicity of the MUC1 expressing 410.4 mammary epithelial tumour cell line no evidence of autoimmunity could be seen in the vaccinated mice. The tumorigenicity of the same cell line expressing only MUC1 or B7.1 was not significantly reduced suggesting that the immunogenic properties of MUC1 are specifically enhanced by B7.1. expression. The effects are related to T cell responses since no difference in tumorigenicity was seen in B7.1/MUC1- expressing cells in the nude mouse, and *in vivo* depletion of CD4+ and CD8+ T cells in the transgenic mice abolished the protective effect of co-expression of MUC1 and B7.1

Methods

Development of 410.4 derived cell lines. The 410.4 cell line was derived from a mammary tumour developing in a Balb/c mouse cross-fostered on an MMTV-carrying C3H mouse (27,28). The 410.4H and E3 cell lines were developed from the 410.4 cells (25) by transfection of a plasmid expressing the hygromycin resistance gene (410.4H) or with this plasmid together with a MUC1 expressing plasmid (E3). Calcium phosphate transfection of 410.4H and E3 cells was perfomed using the pbabe-neo plasmid expressing the neomycin resistance gene (kind gift of H. Land, ICRF, London) with or without the cDNA encoding murine B7.1 contained in the plasmid πLN (a kind gift from P. Linsley, Seattle). After selection with neomycin, clones were isolated, and the nomenclature of those used in the experiments described here is illustrated in Figure 1. The E3 cell line and transfectants thereof express the epitope in the MUC1 tandem repeat recognised by the antibody SM3 which is selectively expressed in human breast cancers. All the cell lines were subsequently grown in Dulbecco's E4 medium containing 600μg/ml G418, 200μg/ml hygromycin and 10% FCS.

Transgenic mice. The transgenic mice homozygous for the MUC1 gene and designated SacII are $H2^k$ (24). These mice were crossed with Balb/c mice to produce F1 hybrids (haplotype $H\text{-}2^{kd}$) in order to accept the 410.4 tumour cell derivatives (haplotype $H\text{-}2^{d}$).

Growth of cell lines in transgenic mice. SacII transgenic x Balb/c F1 mice aged between 6 and 14 weeks old were randomly split into groups. Each mouse was injected subcutaneously with a suspension of cells in phosphate buffered saline (PBS), from one of the 410.4-derived clones. Experiments performing titrations in SacII Balb/c mice showed that 10^5 was the lowest number cells which consistently induced tumours in the majority of mice injected with either the parental tumour, the clone 410.4-H.neo or with the MUC1 expressing clone E3.neo. Mice were inspected and palpated 2-3 times per week

for the presence of tumour and the health of the animal was assessed. Tumour size was estimated by measuring the largest diameter and its perpendicular, and the mice were euthanised when the largest tumour diameter reached 15mm, the tumour ulcerated, or the mouse appeared to be in distress or sick. Experiments in 6-14 week old nu/nu mice were performed as described above for the transgenic mice. All experiments were conducted according to Home Office Guidelines under an approved project license (JTP)

In vivo depletion of CD4+ and CD8+ cells. Five to six week old SacII x Balb/c F1 mice were thymectomised under general anaesthetic and surviving mice were randomised into one of four groups. One week later they underwent T-cell subset depletion by intravenously injecting the relevant anti-T cell antibodies in a volume of 200 μl of PBS, three times over a five day period (29). CD4+ depletion was achieved by using 1mg of the synergistic pair of antibodies YTS 191.1.2 (a rat IgG2b anti-mouse CD4 antibody, epitope a) and YTA 3.1.2 (a rat IgG2b anti-mouse CD4 antibody, epitope b). CD8+ depletion was achieved by using 1mg of the synergistic pair of antibodies YTS 169.4.2.1 (a rat IgG2b anti-mouse CD8α chain, antibody) and YTS 156.7.7 (a rat IgG2b anti-mouse CD8β chain [or α/β complex] antibody). CD4+/CD8+ depletion was achieved by using a combination of all the four antibodies described above to a total of 2mg of antibody. Mice in the control group received 1mg of the irrelevant antibody PYLT1 (an antibody against the polyoma virus large T antigen) in a volume of 200 μl of PBS. Before injecting the tumour cell lines, some mice were sacrificed in each group and their splenocytes analysed for CD4+/CD8+ expression by flow cytometry. All mice tested had successfully been depleted of the relevant subsets. One week following the T cell depletions each mouse received a subcutaneous injection 1×10^5 E3-B7(Z) cells made up to 200μl with PBS. The mice were then followed as described above for tumour development.

Immunoperoxidase staining of sections. Tumours were fixed in methacarn (60% methanol: 30% chloroform: 10% acetic acid) for 4 hours, washed in 70%

ethanol, and processed for wax-embedding. 5μm sections were dewaxed, endogenous peroxide activity was blocked by incubation in 1% H_2O_2 in methanol (v/v) for 30 minutes, and non-specific antibody binding was blocked by incubating sections in 50% FCS overnight at room temperature. After removing excess blocking serum, biotinylated affinity purified SM3 (50μg/ml) or HMFG-1 (100μg/ml), monoclonal antibodies which recognise epitopes within the core protein repeat (21,30,31), were added for 1 hour. Sections were washed in PBS and incubated in Horse-radish peroxidase (HRP)-conjugated avidin-biotin (ABC complex - Dako Ltd) for 30 minutes. The bound HRP was visualised by incubating in 3-3' diaminobenzidine tetrahydrochloride (200mg in 400ml PBS + 60 μl H_2O_2) for several minutes. Sections were counterstained with haematoxylin, dehydrated, cleared and mounted.

Examination of tissues for evidence of Autoimmunity. One group of 10 mice and 3 groups of 5 mice were given subcutaneous injections into the flank of 1 x 10^5 cells of E3-B7, $2x10^6$ cells of E3-B7, $1x10^5$ cells of E3.neo and PBS respectively. All mice were euthanised on day 12 following the initial injection. The tumours and organs were analysed histologically for lymphocyte infiltration and evidence of tissue destruction.

Flow cytometry. All cell clones were screened for membrane expression of murine B7.1 by staining with CTLA4-Ig (32) which consists of the extracellular part of the murine CTLA4 protein linked to the constant region of human IgG1. Monolayer cells were trypsinized, washed in PBS and a minimum of 5 x 10^5 cells were resuspended in 100 μl of the neat supernatant from the CTLA4-Ig hybridoma. After 1 hour incubation on ice, cells were washed 3 times in cold PBS and resuspended in 100 μl of fluorescein isothiocyanate (FITC) conjugated rabbit anti-human antibody (DAKO™, Denmark diluted 1:40) and incubated on ice in the dark for 1 hour. After washing as above, cells were resuspended in 500μl of PBS and 5000 cells were analysed by a Becton-Dickinson FACScan flow cytometer. The cell line which produces the CTLA4-Ig was a kind gift of Dr. P. Lane, Basle. The clones were also screened for

expression of MUC1 by incubating with neat supernatant from the HMFG-1 hybridoma as described above, followed by a FITC conjugated rabbit anti-mouse Ig second antibody (DAKO™, Denmark). HMFG-1 is directed against PDTR in the immunodominant tandem repeat region of MUC1.

Cytotoxic T cell assays. SacII transgenic x Balb/c F1 mice were immunised, in the flank, with $1x10^5$ cells of the B7/MUC1 expressing tumour E3-B7(Z). Four to five weeks later splenocytes were isolated and resuspended at a concentration of 50×10^6 live spleen cells in 10ml of culture medium (IMDM, 10% FCS/ 5μm 2-β-mercaptoethanol). The splenocytes were stimulated *in vitro* with 1×10^6 of mitomycin-C treated P815-MUC1 cells and after 5 days, the spleen cells were tested for their ability to lyse P815-MUC2 cells in a standard, chromium release CTL assay(33). Briefly, 5×10^4 ^{51}Chromium labelled target cells (P815-MUC1 or the control line P1HTR expressing only the hygromycin resistance marker) were mixed with effector lymphoctes at effector: target ratios from 50:1 to 1.5:1 in a 96-well round bottomed plate. Control samples to which no lymphoctes had been added, indicated the level of spontaneous lysis and samples to which 100μl of 10% Triton x100 were added indicated maximum lysis. The percentage of specific lysis was determined as: (mean sample cpm - mean spontaneous cpm)/ (mean maximum cpm- mean spontaneous cpm)

Statistical analyses. Survival was assessed using the logrank test for heterogeneity and Kaplan-Meier survival curves. The growth of tumour cells was assessed using the Kruskal-Wallis test. All analyses were performed using Stata™.

Results

Co-expression of MUC1 and B7.1 in a mouse mammary cell line inhibts tumour growth in MUC1 transgenic mice

The tumour cell line used to express the MUC1 antigen is the 410.4 mammary epithelial cell, originally cultured from a mammary tumour developing in a Balb/c mouse cross fostered on an MMTV carrying mouse strain (27,28). The E3 cell line derived from the 410.4 cell line expressing MUC1 and hygromycin (used for selection) and the control 410.4 transfectant expressing only the selectable marker (410.4-H) have been previously described (25). These 2 cell lines were transfected with the mouse B7.1 gene together with the neomycin resistance gene or only with the selectable marker. The cell lines developed and their nomenclature is shown in Figure 1. As shown in Figure 2, the three cell lines expressing both MUC1 and B7.1 express different levels of B7.1 with E3-B7(Z) showing the highest level and E3-B7(X) the lowest expression. Figure 2 also shows that the level of expression of B7.1 is comparable in the the E3-B7(Z) cell line and in the cell line not expressing MUC1 (410.4-HB7). Moreover, the levels of expression of MUC1 in the B7.1 tranfectants are the same as those in the parental E3 cell line and in E3 neo transfectant (see Figure 3). The growth rates of all of the cell lines *in vitro* were not significantly different (data not shown).

Although the sequence of the human MUC1 gene shows considerable homology with the mouse homologue (muc1) in regions outside of the tandem repeat, the sequence of the tandem repeat shows only 40% homology(23). In particular the sequence APDTRPA between glycosylation sites is different. Since both the humoral and CTL response in mice is primarily to the tandem repeat (2,19-22) the antibodies and specific T cells induced in mice would not be expected to react with the normal epithelial cells of the mouse expressing the mouse (but not the human) mucin. The transgenic mouse expressing the human MUC1 gene on normal glandular epithelial tissues (24) provides a more appropriate model for predicting the immunogenicity of the MUC1 antigen in humans, and assessing the

relationship between tolerance and autoimmunity. To accept the 410.4 tumour derived cell lines, which are H2d, the homozygous MUC1 transgenic mice (designated SacII), which are H2k, were crossed with Balb/c mice and F1 females were used for assesssing tumorigenicity of the cell lines.

Figure 4 shows the Kaplan-Meier survival curves for mice given the four cell lines 410.4-H.neo , 410.4-B7, E3-neo amd E3-B7(Z) at three different doses (10^5 [figure 4A], 2×10^5 [figure 4B], 10^6 [figure 4C]), and the data is summarised in Table 1. Figure 5 shows the growth rates for the inividual tumours at the higher dose. The data of figure 4 and Table 1 show that the expression of the co-stimulatory molecule together with the MUC1 antigen has a highly significant effect on survival at the two lower challenge doses, while expression of either B7.1 or MUC1 alone has no such effect. Moreover, even though none of the mice survived the challenge dose of 10^6 cells per mouse, there was a strongly significant effect of co-expression of B7.1 and MUC1 on the growth rate of the tumours resulting in a marked delay in the time of death.

Influence of level of expression of B7.1

The influence of the level of expresson of the B7.1 molecule was examined by following the development of tumours in groups of mice injected with the three cell lines showing different levels of B7.1 expression, as determined by FACS analysis (see figure 1B). Figure 6 shows the Kaplan Meier survival curve for the mice in the three groups as compared to that for mice given the cell line expressing only MUC1 (E3 -H.neo). Clearly to affect the growth of the MUC1 expressing tumour, the level of B7 expression needs to be relatively high. Since the strongest effects were seen with the E3-B7(Z) cell line, this cell line was used in subsequent experiments.

Growth of the cell lines in nude mice

Since the 410.4 cell line and its transfectants express Class I molecules at the surface, they should not be susceptible to killing by NK cells which are

inhibited by Class I expression. Although nude mice lack T cells, they have higher than normal levels of NK cells. The growth of the cell lines was examined in Balb/c nu/nu mice by injecting 10^5 cells of 410.4-B7, 410.4-H.neo, E3, or E3-B7(Z), with 8 mice per group. Figure 7 shows the survival curves (A) and the growth curves (B) for the individual mice, and it can be seen that expression of MUC1 with or without B7.1 at any level has no effect on the growth of the 410.4 tumour cell line in these mice. These results suggest that T cell responses are involved in the decrease in tumorigenicity of the E3-B7 cells in the MUC1 transgenic mice

T Cell responses

Attempts were were made to induce cytotoxic T cells using both the 410.4 derived lines and a syngeneic cell line (P815) expressing MUC1 (33,34). Using the P815 MUC1 line to stimulate T cells from the spleens of the injected mice, a low level of specific killing was observed when the same cell line was used as a target. Although the lysis seen was not very high, the killing observed was specific since untransfected P815 cells were not killed. An example of this effect is shown in figure 8 using cells cultured from spleens taken from mice injected with E3-B7(Z) cells. T cell proliferative responses were not seen using the tandem repeat peptide or the MUC1 transfected 410.4. cell lines to stimulate spleen cells taken from mice at different times after injection with the transfected cells, even when dendritic cells cultured from bone marrow(35) were used to present the tandem repeat peptide.

A synthetic peptide covering 3 tandem repeats are available covering was used to measure antibodies in the serum of mice given the various cell lines. The strongest and most consistent antibody response was seen in the mice given the higher doses (10^6 cells) of the E3-B7(Z)cells (data not shown). However, at the lower doses the antibody response measured in this way was not consistent.

In vivo depletion of CD4+ and/or CD8+ cells stimulates tumorigenicity of E3-B7(Z) cells in the MUC1 trangenic mice.

To assess the importance of T cell responses *in vivo* in the inhibition of tumorigenicity induced by expressing both MUC1 and B7.1 in 410.4 cells, mice were depleted of CD4+ or CD8+ cells or of both subclasses by injecting antibodies to the CD4 or CD8 molecules after thymectomy(ref and see Materials and methods). Control mice were thymectomised but not treated with antibodies. The survival curves and growth of individual tumours are shown in Figures 10A and B. Although thymectomy itself showed some inhibitory effect on the ability of the mice to resist the growth of the tumour cells, the effect of depletion of either CD4+ cells or CD8+ cells had a much more pronounced effect, and survival was decreased and rate of tumour growth increased: The effect on both survival and growth rate was marginally stronger in the CD4 depleted mice, however, the mice depleted of both CD4+ and CD8+ cells showed the worst survival. These results demonstrate unequivocally that the protection afforded by expression of B7.1 together with the MUC1 antigen is operating through stimulation of specific CD8+ and CD4+ T cells. Thus even though the MUC1 antigen is expressed as a self antigen in the transgenic mice, tolerance can be overcome and an effective immune response can be induced.

Enhancement of immunogenicity does not induce auto-immunity.

The MUC1 mucin is expressed on most glandular epithelial cells including the pancreas, stomach, lung , kidney and the lactating breast, and this pattern of expressio is faithfully reprodcued in the MUC1 transgenic mice. However, the glycoforms expressed by the normal tissues are not the same as the glycoforms expressed by malignant mammary epithelial cells where the mucin is underglycosylated and reacts with the antibody SM3 (30,36,37). The mice injected with the E3-B7(Z) cells survived their natural life span and tissues were taken for examination at various times after being injected with the tumour cells. An examination of the normal glandular epithelial tissues expressing MUC1 showed no evidence of abnormalities or lymphocyte infiltrates, confirming that the immune response which prevented the growth of the tumour cells did not adversely affect the normal tissues.

Discussion

Pre-clinical testing of therapeutic agents for cancer patients requires model systems which can give an indication of both efficacy and potential toxicity. In the case of chemotherapeutic agents, the efficacy and toxicity both relate to the same action of the drug in inhibiting the proliferation of dividing cells and "maximum tolerated doses" are estimated to avoid lethal side effects. Preclinical testing of antigens for use in immunotherapy is more difficult, particularly where the antigen in humans is a self antigen and could give rise to auto-immunity. In this paper we have used a transgenic mouse expressing the human MUC1 mucin as a self antigen to determine if tolerance to MUC1 can be overcome and an effective immune response generated without the induction of autoimmunity. The data also suggest that the MUC1 antigen requires co-stimulation generated by the B7.1 T cell interaction to induce an effective response, analogous to the report of Cayeux et al (38). Alternatively, antigen presentation occurred through cross-priming of tumour specific T cells and was stimulated by B7.1 expression (39).

The MUC1 antigen is normally expressed on the luminal surface of most glandular epithelial cells, but is up-regulated and aberrantly glycosylated in breast and other carcinomas (1). Several clinical studies are in progress using MUC1 based vaccines mostly in breast cancer patients with advanced disease (9-12). MUC1 is a type I membrane glycoprotein with the external domain made up of 25-100 tandem repeat units, depending on the allele. The molecule is highly repetitive with an extended structure due to the addition of multiple O-glycans to serines and threonines found in the tandem repeat sequence. In cancer, the O-glycans are shorter (37) so that core protein epitopes are exposed which are normally masked and novel carbohydrate epitopes appear (36,40). A similar change appears to occur in mice, since the same epitope in the tandem repeat (recognised by the antibody SM3 is masked in normal tissue in the MUC1 transgenic mouse (24) but exposed in tumours developing in these mice (26). This change in the glycosylation will affect any immune response which depends on the interaction of the whole MUC1 molecule with an effector cell. Moreover, in addition to B cell

responses, MHC unrestricted T cell responses have been reported which are specific for the cancer -associated mucin and which appear to involve multiple low affinity reactions of MUC1 with the T cell receptor (6,7). Interactions with surface lectins on antigen presenting cells (APCs) will also depend on the glycoform as will the breakdown in the APC and the presentation by MHC Class II (41). With MUC1 therefore there are multiple ways of recruiting immune responses in addition to the classical stimulation of T cells by specific peptides presented by MHC molecules. However, Class I epitopes have also been reported which can be presented by both mouse Class I molecules and by the human HLA A2 allele (20,42). It is therefore of interest to ask whether the immune responses which lead to effective tumour rejection are those which require the co-stimulatory signals generated by the interaction of B7.1 with T cells. Our results clearly show that expression of B7.1 together with the MUC1 mucin in a mouse mammary tumour cell dramatically enhances its immunogenicity and that the immunity requires the activity of CD8+ and CD4+ T cells. Over-expression of B7.1 in the absence of MUC1 had no significant effect on the immunogenicity of the mammary epithelial tumour cells (410.4) used in the experiments. The data suggest that, even if non-classical T cell responses to MUC1 do occur, these are unlikely to be generated by the tumour cells themselves, which do not express co-stimulatory molecules and that, as with other antigens, presentation by professional antigen presenting cells is required, or the tumour cells need to be manipulated to express co-stimulatory molecules. The data also show that although MUC1 is present on normal tissues, these tissues are not attacked by the cells which reject the MUC1 expressing tumour cells, thus going some way to establishing the safety of vaccines based on MUC1.

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References

1. Taylor-Papadimitriou, J., L. Stewart, J. Burchell, and P. Beverley. 1993. The polymorphic epithelial mucin as a target for immunotherapy. *Ann. N. Y. Acad. Sci..* 690:69-79.
2. Gendler, S., J. Taylor-Papadimitriou, T. Duhig, J. Rothbard, and J. Burchell. 1988. A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. *J. Biol. Chem.* 263:12820-3.
3. Wandhall, H.H., H. Hassan, K. Mirgorodskaya, A.K. Kristensen, P. Roepstorff, E.P. Bennett, P.A. Nielsen, M.A. Hollingsworth, J. Burchell, J. Taylor-Papadimitriou, H. Clausen. 1997. Susbstrate specificities of three members of the human UDP-N-Acetyl-∝-D-galactosamine: Polypeptide N-Acetylgalactosaminyl-transferase family, GalNAc-T1, -T2 and -T3. *J. Biol. Chem.* 272(38): 23503-23514.
4. Burchell, J. and J. Taylor-Papadimitriou. 1993. Effect of modification of carbohydrate side chains on the reactivity of antibodies with core-protein epitopes of the MUC1 gene product. *Epith. Cell. Biol.* 2: 155-162.
5. von Mensdorff-Pouilly, S., M.M. Gourevitch, P. Kenemans, A.A. Verstraeten, S.V. Litvinov, G.J. van Kamp, S. Meijer, J. Vermorken, and J. Hilgers. 1996. Humoral immune response to polymorphic epithelial mucin (MUC1) inpatients with benign and malignant breast tumours. *Eur. J. Cancer* . 32: 325-1331.
6. Ioannides, C.G., B. Fisk, K.R. Jerome, T. Irimura, J. Taylor Wharton, and O.J. Finn. 1993. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. *J. Immunol.* 151:3693-3703.
7. Barnd, D.L., M.S. Lan, R.S. Metzgar, and O.J. Finn. 1989. Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. *Proc. Natl. Acad. Sci. U.S.A.* 86:7159-63.
8. Reddish, M.A., G.D. MacLean, R.R. Koganty, J. Kanmitchell, V. Jones, M.S. Mitchell, B.M. Longenecker. 1998. Anti-MUC1 class I restricted

CTLs in metastatic breast cancer patients immunised with a synthetic MUC1 peptide. *Int. J. Cancer.* 76(6): 817-823.

9. MacLean, G.D., M.B. Bowen Yacyshyn, J. Samuel, A. Meikle, G. Stuart, J. Nation, S. Poppema, M. Jerry, R. Koganty, T. Wong, et. al. 1992. Active immunization of human ovarian cancer patients against a common carcinoma (Thomsen-Friedenreich) determinant using a synthetic carbohydrate antigen. *J. Immunother.* 11:292-305.

10. Miles, D.W., K.E. Towlson, R. Graham, M. Reddish, B.M. Longenecker, J. Taylor-Papadimitriou, R.D. Rubens. 1996. A randomised phase II study of sialyl-Tn and DETOX-B adjuvant with or without cyclophosphamide pretreatment for the active specific immunotherapy of breast cancer. *Br. J. Cancer. 74: 1292-1296.*

11. Scholl, S., B. Acres, M. Kieny, J. Balloul, A. Vincent-Salomon, L. Deneux, E. Tartour, H. Fridman, P. Pouillart. 1997. The polymorphic epithelial mucin (MUC1): a phase I clinical trial testing the tolerance and immunogenicity of a vaccinia virus-MUC1-IL2 construct in breast cancer. *Breast Cancer Treatment & Research* 46: 268 (Abstr).

12. Finn, O.J., K.R. Jerome, R.A. Henderson, G. Pecher, N. Domenech, J. Magarian-Blander, S.M. Barratt-Boyes. 1995. MUC-1 epithelial tumour mucin-based immunity and cancer vaccines. *Immunological Reviews.* 145: 61.89.

13. Chen, L., S. Ashe, W.A. Brady, I. Hellstrom, K.E. Hellstrom, J.A. Ledbetter, P. McGowan, and P.S. Linsley. 1992. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell.* 71:1093-102.

14. Hodge, J.W., S. Abrams, J. Schlom, and J.A. Kantor. 1994. Induction of antitumor immunity by recombinant vaccinia viruses expressing B7-1 or B7-2 costimulatory molecules. *Cancer Res.* 54:5552-5.

15. Martin Fontecha, A., F. Cavallo, M. Bellone, S. Heltai, G. Iezzi, P. Tornaghi, N. Nabavi, G. Forni, P. Dellabona, and G. Casorati. 1996. Heterogeneous effects of B7-1 and B7-2 in the induction of both protective and therapeutic anti-tumor immunity against different mouse tumors. *Eur. J. Immunol..* 26:1851-9.

16. Ramarathinam, L., M. Castle, Y. Wu, and Y. Liu. 1994. T cell costimulation by B7/BB1 induces CD8 T cell-dependent tumor rejection: an important role of B7/BB1 in the induction, recruitment, and effector function of antitumor T cells. *J. Exp. Med.* 179:1205-14.

17. Townsend, S.E., and J.P. Allison. 1993. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science.* 259:368-70.

18. Yang, G., K.E. Hellstrom, I. Hellstrom, and L. Chen. 1995. Antitumor immunity elicited by tumor cells transfected with B7-2, a second ligand for CD28/CTLA-4 costimulatory molecules. *J. Immunol.* 154:2794-800.

19. Apostolopoulos, V., P.X. Xing, and I.F. McKenzie. 1994. Murine immune response to cells transfected with human MUC1: immunization with cellular and synthetic antigens. *Cancer Res.* 54:5186-93.

20. Apostolopoulos, V., B.E. Loveland, G.A. Pietersz, I.F.C. McKenzie. 1995. CTL in mice immunised with human Mucin 1 are MHC-restricted. *J. Immunol.* 155: 5089-5094.

21. Burchell, J., J. Taylor Papadimitriou, M. Boshell, S. Gendler, and T. Duhig. 1989. A short sequence, within the amino acid tandem repeat of a cancer-associated mucin, contains immunodominant epitopes. *Int. J. Cancer.* 44:691-6.

22. Taylor-Papadimitriou, J. 1991. Report on the First International Workshop on Carcinoma-associated Mucins. *Int. J. Cancer* . 49: 1-5.

23. Spicer, A.P., G. Parry, S. Patton, and S.J. Gendler. 1991. Molecular cloning and analysis of the mouse homologue of the tumor-associated mucin, MUC1, reveals conservation of potential O-glycosylation sites, transmembrane, and cytoplasmic domains and a loss of minisatellite-like polymorphism. *J. Biol. Chem.* 266:15099-109.

24. Peat, N., S.J. Gendler, N. Lalani, T. Duhig, and P.J. Taylor. 1992. Tissue-specific expression of a human polymorphic epithelial mucin (MUC1) in transgenic mice. *Cancer Res.* 52:1954-60.

25. Lalani, E.N., F. Berdichevsky, M. Boshell, M. Shearer, D. Wilson, H. Stauss, S.J. Gendler, and P.J. Taylor. 1991. Expression of the gene coding

for a human mucin in mouse mammary tumor cells can affect their tumorigenicity. *J. Biol. Chem.* 266:15420-6.

26. Graham, R.A., L.S. Stewart, N.P. Peat, P. Beverley, J. Taylor-Papadimitriou. 1995. MUC1-based immunogens for tumour therapy: Development of murine model systems. *Tumour Targeting.* 1: 211-221.

27. Miller, B.E., F.R. Miller, J. Leith, and G.H. Heppner. 1980. Growth interaction in vivo between tumor subpopulations derived from a single mouse mammary tumor. *Cancer Res.* 40:3977-81.

28. Miller, F.R., B.E. Miller, and G.H. Heppner. 1983. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. *Invasion Metastasis.* 3:22-31.

29. Baskar, S., S. Ostrand-Rosenberg, N. Nabavi, L.Nadler, G. Freeman, and L. Glimcher. 1993. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. *Proc. Natl. Acad. Sci. USA* 90:5687-5690

30. Burchell, J., S. Gendler, J. Taylor Papadimitriou, A. Girling, A. Lewis, R. Millis, and D. Lamport. 1987. Development and characterization of breast cancer reactive monoclonal antibodies directed to the core protein of the human milk mucin. *Cancer Res.* 47:5476-82.

31. Taylor-Papadimitriou, J., J. A. Peterson, J. Arklie, J. Burchell, R.L. Ceriani, and W.F. Bodmer. 1981. Monoclonal antibodies to epithelium-specific components of the human milk fat globule membrane: production and reaction with cells in culture. *Int. J. Cancer.* 28:17-21.

32. Lane P., W. Gerhard, S. Hubele, A. Lanzavecchia, and F. McConnell. 1993. Expression and functional properties of mouse B7/BB1 using a fusion protein between mouse CTLA4 and human gamma 1, *Immunology.* 80: 56-61.

33. Graham R.A., J.M. Burchell, P. Beverley, and J. Taylor-Papadimitriou. 1996. Intramuscular immunisation with *MUC1* cDNA can protect C57

mice challenged with MUC1-expressing syngeneic mouse tumour cells. *Int. J. Cancer.* 65: 664-670.

34. Acres, B., M. Hareuveni, J-M. Balloul, and M-P. Kieny. 1993. Vaccinia virus MUC1 immunisation of mice: immune response and protection against murine tumours bearing the MUC1 antigen. *J. Immunother.* 14: 136-143.

35. Inaba, K., M. Inaba, N. Romani, et. al. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176: 1693.

36. Girling, A., J. Bartkova, J. Burchell, S. Gendler, C. Gillett, and J. Taylor-Papadimitriou. 1989. A core protein epitope of the polymorphic epithelial mucin detected by the monoclonal antibody SM-3 is selectively exposed in a range of primary carcinomas. *Int. J. Cancer.* 43:1072-1076.

37. Lloyd, K.O., J. Burchell, V. Kudryashov, B.W.T. Yin, J. Taylor-Papadimitriou. 1996. Comparison of O-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines. *J. Biol. Chem.* 271(52): 33325-33334.

38. Cayeux, S., G. Richter, G. Noffz, B. Dorken, T. Blankenstein, T. 1997. Influence of gene-modified (IL-7, IL-4, and B7) tumour cell vaccines on tumour antigen presentation. *J. Immunol.* 158: 2834-2841.

39. Huang, A., A. Bruce, D. Pardoll, H. Levitsky. 1996. Does B7-1 expression confer antigen-presenting cell capacity to tumours in vivo? *J. Exp. Med.*183: 769- 776.

40. Reddish, M.A., J. Jackson, R.R. Koganty, D. Qiu, W. Hong, B.M. Longenecker. 1995. Specificites of anti-sialyl-Tn and anti-Tn monoclonal antibodies generated using novel clustered synthetic glycopeptide epitopes. *Glycoconjugate J.*

41. Galli-Stampino, L., E. Meinjohanns, K. Frische, M. Meldal, T. Jensen, O. Werdelin, S. Mouritsen. 1997. T-cell recognition of tumour-associated carbohydrates: The nature of the glycan moiety plays a decisive role in determining glycopeptide immunogenicity. *Cancer Res.* 57: 3214-3222.

42. Apostopoulos, V., V. Karanikas, J.S. Haurum, I.F.C. McKenzie. 1997. Induction of HLA-A2-restricted CTLs to the mucin 1 human breast cancer antigen. *J. Immunol.* 159:5211-5218.

Figure Legends

Figure 1 Development and nomenclature of 410.4 derived cell lines transfected with MUC1, B7.1 and selectable markers. For details of transfection and clone isolation see methods.

Figure 2 Surface expression of B7.1 on the B7.1 transfected lines 410.4-B7, E3-B7(X), E3-B7 (Y) and E3-B7 (Z) as determined by FACS analysis. CTLA-4 binding (....); control (___).

Figure 3 Surface expression of the MUC1 antigen on E3 neo and E3-B7(Z) as determined by FACS analysis.

Figure 4 Kaplan-Meir survival curves of mice injected with various doses of 410.4 derived lines. F1 hybrid mice from the MUC1 transgenic mice crossed with Balb/c mice were injected with 10^5 (A) $2x10^5$ (B) or 10^6 (C) cells of the 410.4-H.neo (4), 410.4-B7 (3), E3-neo (2) or E3-B7(Z) (1) cell lines, and the development of tumours followed as described in Materials and methods. The curves represent composites of several experiments, and P values for the log rank heterogeneity test were < 0.0001 (A), < .02 (B) and <.03 (C). Table 1 summarizes the survival data.

Figure 5 Effect of co-expression of MUC1 and B.7.1 on tumour growth in mice given 10^6 cells (experiment shown in Figure 4C). Group 1, E3-B7(Z); Group 2, E3-neo; Group 3, 410.4-H.B7; Group 4, 410.4-Hneo. Ten mice were injected in each group. Using the Krustal Wallis test for equality of populations, p=0.0001.

Figure 6 Influence of level of expression of B7.1 on the survival of mice injected with E3 cells expressing different levels of B7.1. SacII Balb/c F1 hybrid mice were injected with 10^5 E3-neo cells (4), or one of the three B7.1 expressing E3 cells, E3-B7(X) (3), E3-B7(Y) (2) and E3-B7 (Z) (1) (using 15 mice

per group) and the mice were followed for development of tumours. Log rank test for heteogeneity shows p=<0.01.

Figure 7 Growth of 410.4 derived lines in nu/nu mice. 10^5 cells from each of the cell lines indicated were injected into the mice, and the development of individual tumours followed. **A.** Survival curves for B7(Z) (1), E3-B7(Y) (2), E3-B7(X) (3), 410.4-Hneo (4) 410.4-H.B7 (5) and E3.Neo (6). **B.** Growth of individual tumours. The log rank test for heterogeneity of the survival curve gives a probability of 0.86, and the Krustal-Wallis test for equality of populations gives a probability of 0.98.

Figure 8 F1 hybrid mice were injected with 1 x 10^5 E3-B7(Z) cells and after 4 weeks spleens were removed and CTL responses to MUC1 were analysed. Spleen cells were stimulated with the P815 MUC1 cell line and lysis of P815 MUC1 (Δ-Δ) or P815 control targets (□ - □) and killing measured in a Chromium release assay.

Figure 9 In vivo depletion of $CD4^+$ and $CD8^+$T cells abolishes protective effect of B7.1 expression on tumourigenesis. F1 SacII Balb/c mice were thymectomised and treated with antibodies to CD4 (1) to CD8 (2) or to both antigens (3) or with an irrelevant antibody (4), as described in materials and methods, before challenging with 10^5 E3-B7(Z) cells .The survival of the mice **(A)**, and development of tumours **(B)** was monitored.

Using the log rank test for heterogeneity p=0.027

Table 1. Survival analysis of TG18 x BALB/c F1 mice receiving tumour injections of different clones at doses of A.) 1 x 10^5, B.) 2 x 10^5, C.) 1 x 10^6, cells per mouse. (see Figure 3)

	Deaths / number of mice in experiment		
	Experiment A	*Experiment B*	*Experiment C*
E3-B7(z)	3/18	4/15	10/10
E3-H.neo	13/19	9/10	10/10
410.4H.neo	16/20	10/15	10/10
410.4H.B7	16/20	13/15	10/10
P value	<0.0001	<0.02	<0.03

[P values as calculated for the logrank heterogeneity test for each experiment].

Figure 1

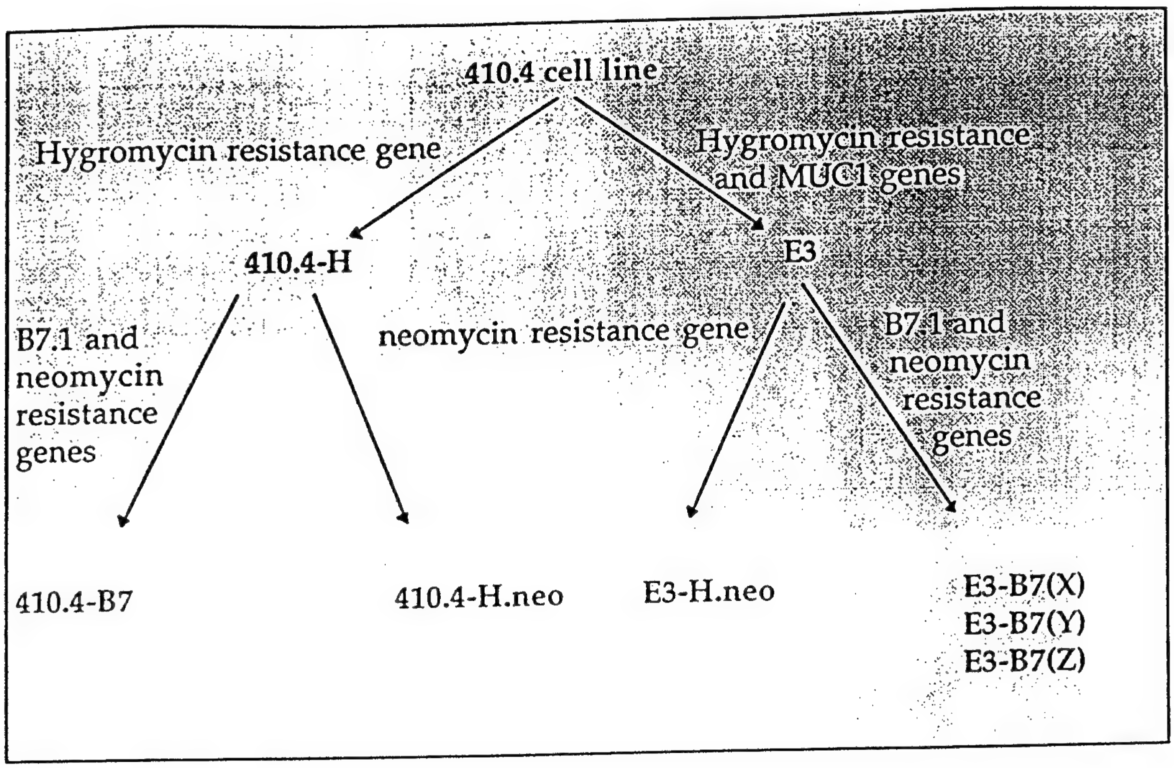

410.4 cell line
Hygromycin resistance gene
Hygromycin resistance and MUC1 genes
410.4-H
E3
B7.1 and neomycin resistance genes
neomycin resistance gene
B7.1 and neomycin resistance genes
410.4-B7
410.4-H.neo
E3-H.neo
E3-B7(X)
E3-B7(Y)
E3-B7(Z)

Figure 2

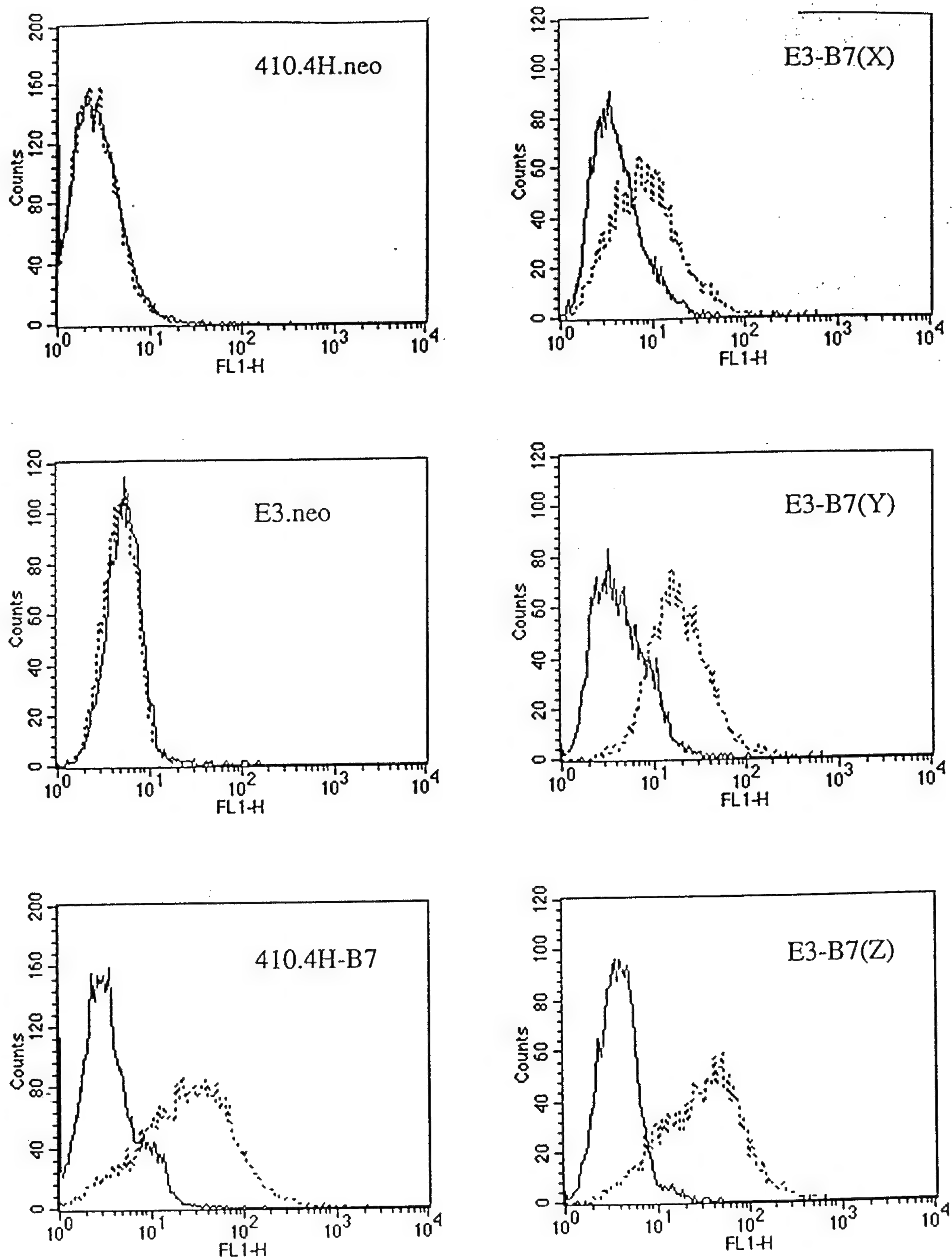
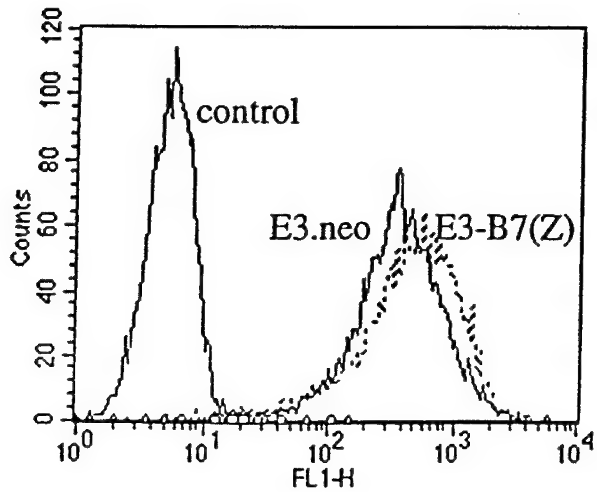


Figure 3



control
E3.neo
E3-B7(Z)
Counts
0
20
40
60
80
100
120
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 4

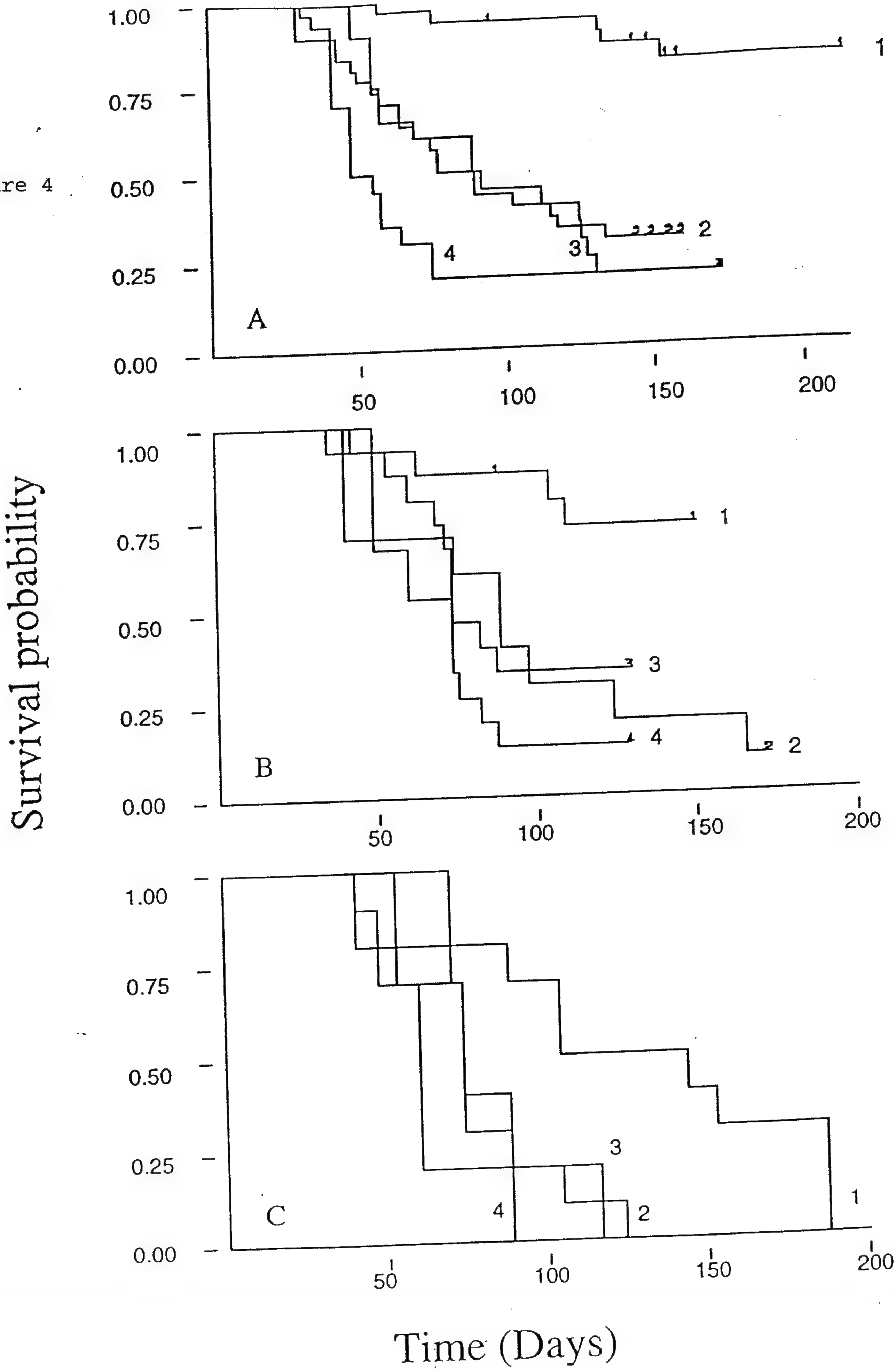

Survival probability
1.00
0.75
0.50
0.25
0.00
A
1
2
3
4
50
100
150
200
B
C
Time (Days)

Figure 5

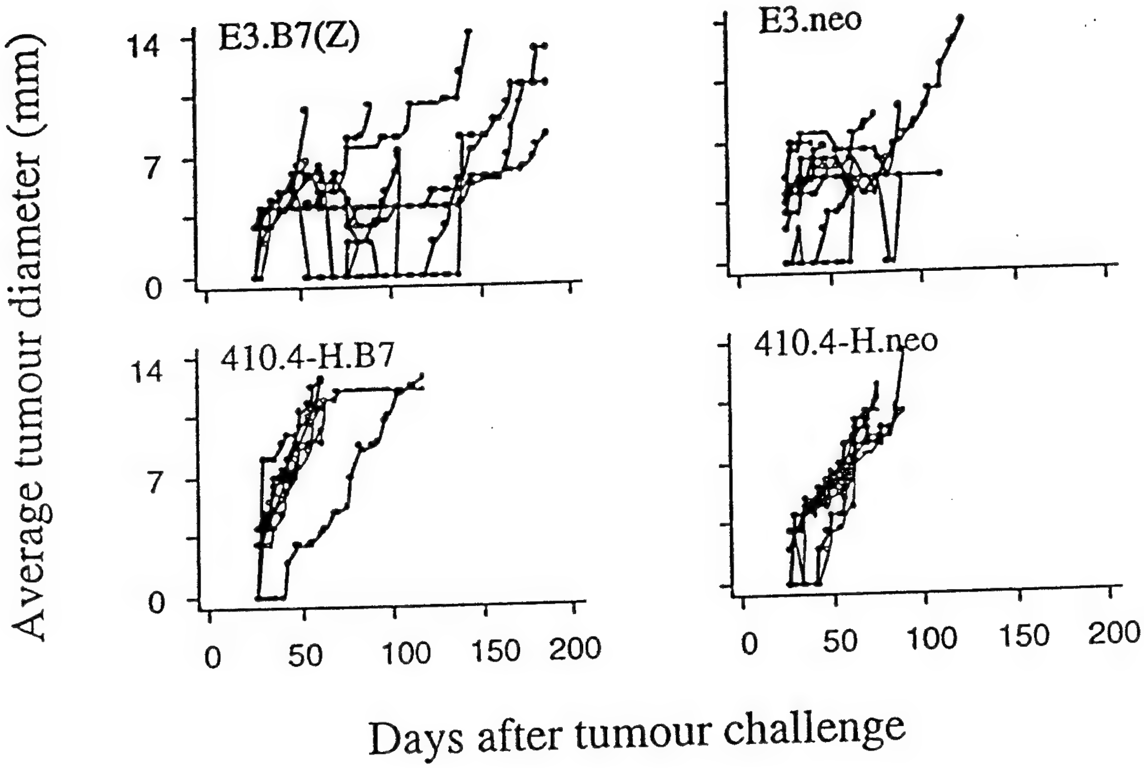
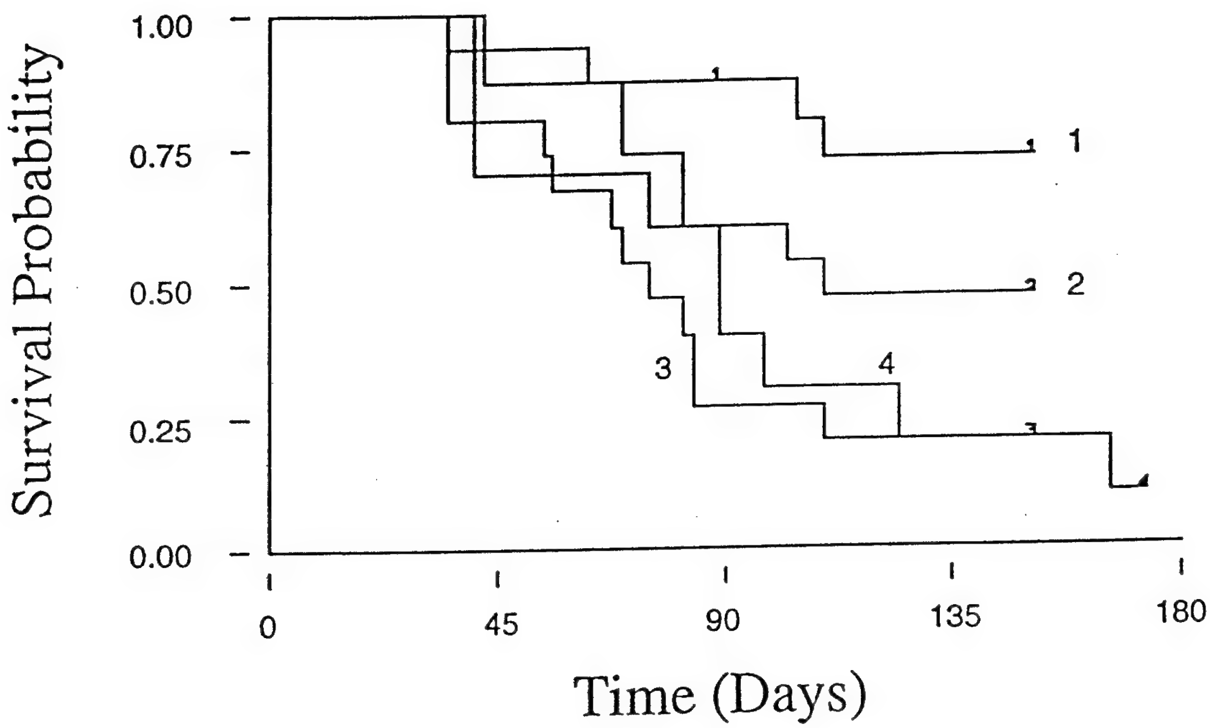

E3.B7(Z)
E3.neo
410.4-H.B7
410.4-H.neo
14
7
0
0
50
100
150
200
Average tumour diameter (mm)
Days after tumour challenge

Figure 6



Survival Probability
1.00
0.75
0.50
0.25
0.00
1
2
3
4
0
45
90
135
180
Time (Days)

Figure 7A

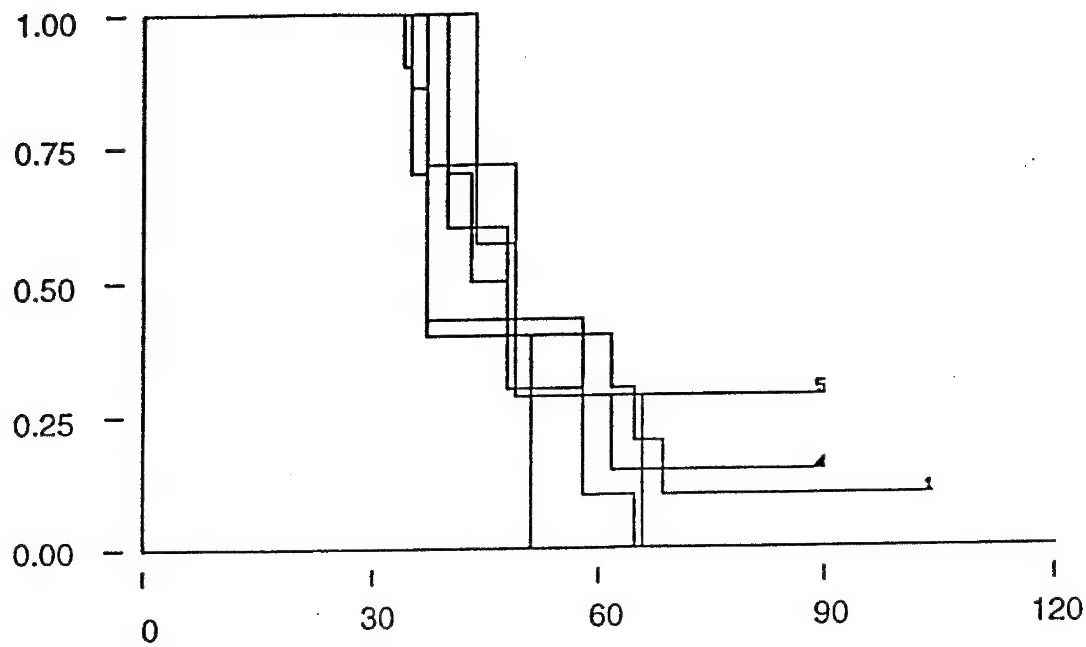
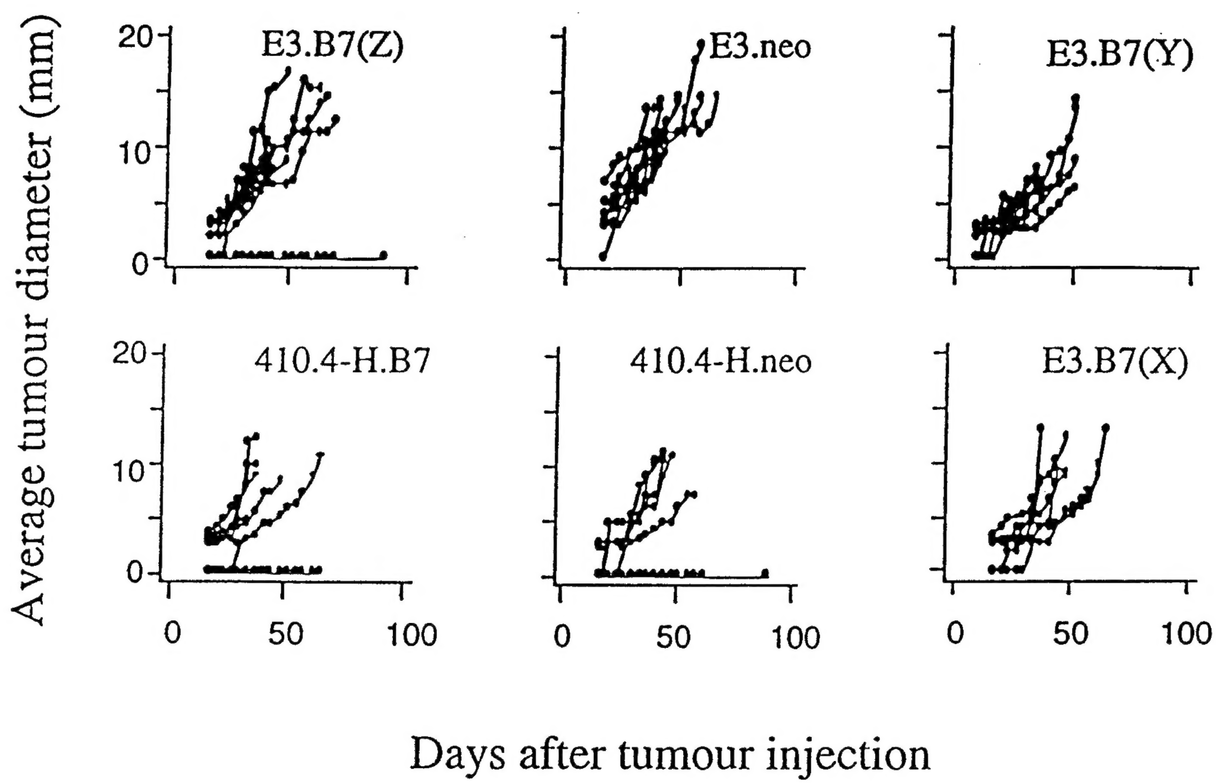
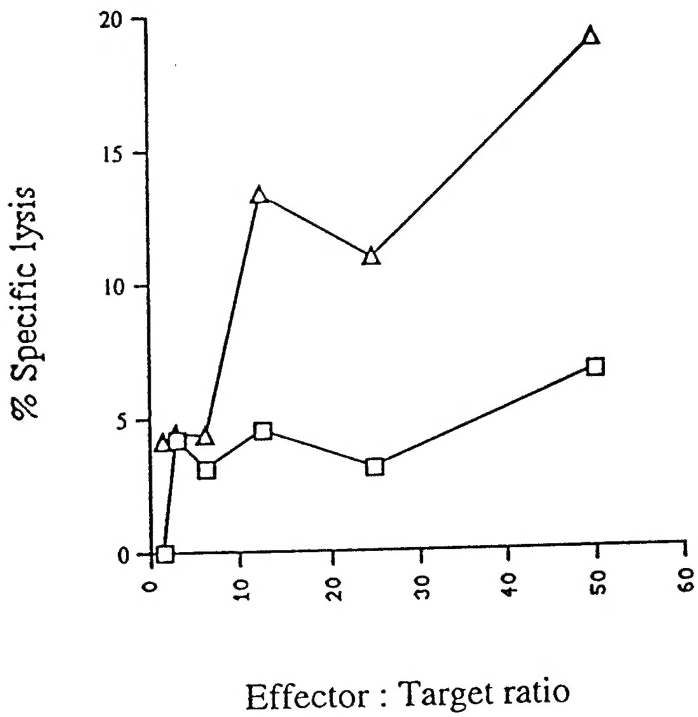


Figure 7B



Average tumour diameter (mm)
E3.B7(Z)
E3.neo
E3.B7(Y)
410.4-H.B7
410.4-H.neo
E3.B7(X)
20
10
0
0
50
100
Days after tumour injection

Figure 8



20
15
10
5
0
% Specific lysis
0
10
20
30
40
50
60
Effector : Target ratio

Figure 9A

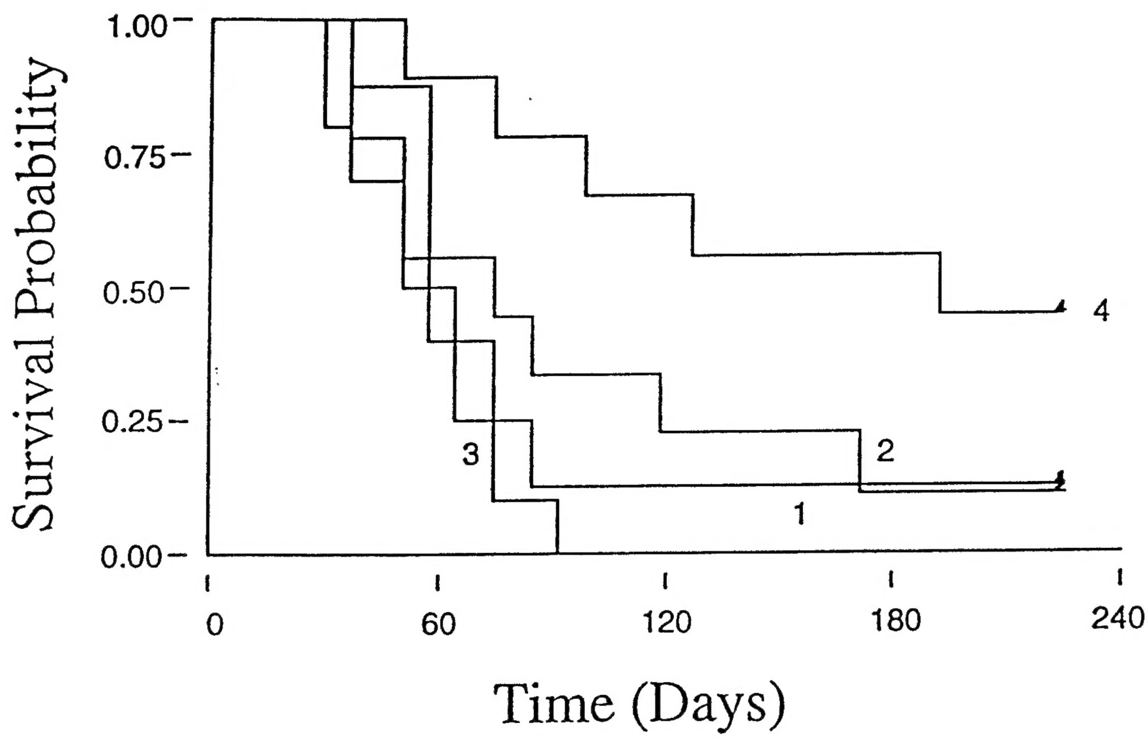
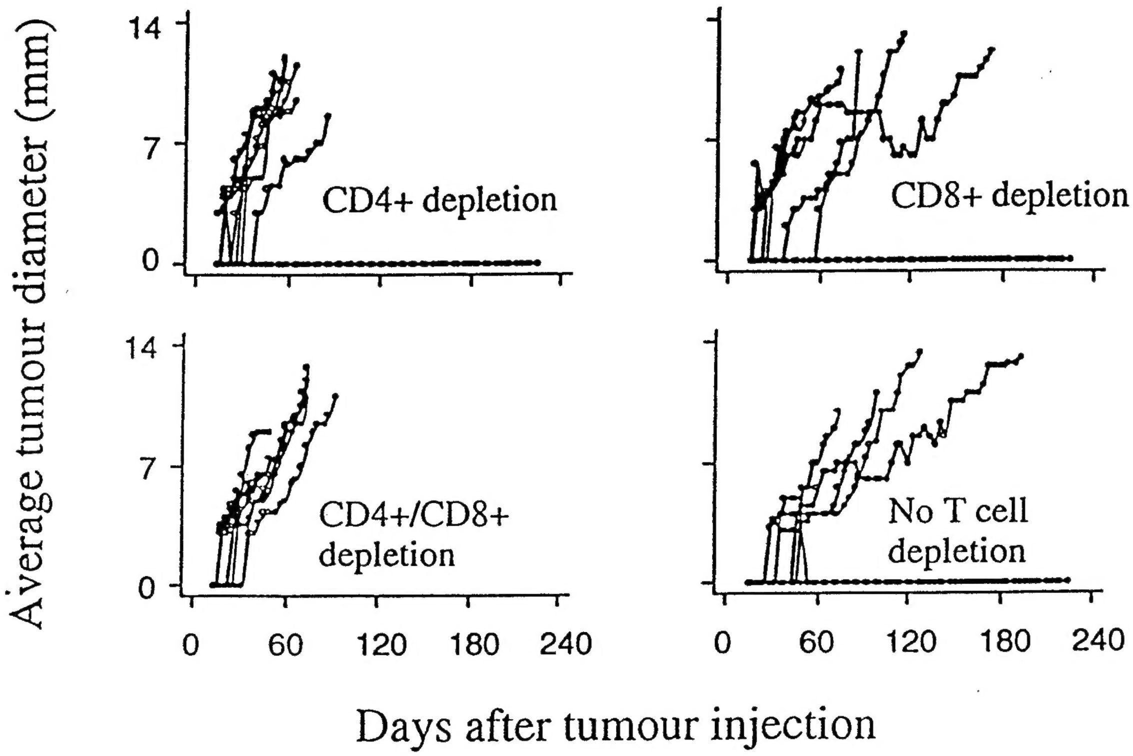

Survival Probability
1.00
0.75
0.50
0.25
0.00
0
60
120
180
240
Time (Days)
1
2
3
4

Figure 9B


Average tumour diameter (mm)
14
7
0
CD4+ depletion
CD8+ depletion
CD4+/CD8+
depletion
No T cell
depletion
0
60
120
180
240
Days after tumour injection